

UNIVERSIDADE DE LISBOA
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Sílvia Marina de Jesus Andrade

DISSERTAÇÃO
MESTRADO EM BIOLOGIA MOLECULAR E GENÉTICA

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2014

“O único bem é o conhecimento
e o único mal a ignorância.”
Sócrates (469-399 a.C)

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RESUMO

As vacinas baseadas em DNA plasmídico são uma alternativa mais segura relativamente às tradicionais, podendo ser estes vetores produzidos mais rapidamente e com maior facilidade no geral. *Escherichia coli* é o hospedeiro mais comum para a produção de vacinas de DNA. Contudo estas bactérias gram negativas possuem na sua membrana externa lipopolissacarídeos, uma endotoxina que se liga a um complexo de recetores CD14/TLR4/MD2, ativando a cascata do complemento e que resulta numa reação inflamatória grave. Assim, este trabalho pretende desenvolver uma alternativa mais segura para as vacinas de DNA em bactérias ácido lácticas uma vez que são comumente utilizadas na indústria alimentar, são consideradas como probióticos e usadas para a expressão de proteínas heterólogas.

O crescimento para duas estirpes (*Lactococcus lactis* LMG 19460 e *Lactobacillus plantarum* CCUG 61730) foi otimizado em pequena escala (crescimento em falcon), chegando-se à escala de 1 litro de cultura celular em condições anaeróbias. Para o processo de eletrotransformação só o baseado em NaCl foi eficiente para a transformação de *L. lactis* LMG 19460, não se tendo obtido transformantes para *L. plantarum* CCUG 61730. Neste trabalho construiu-se um plasmídeo modificado por alteração do *Ribosome binding site* (RBS) de forma a aumentar o número de cópias de plasmídeo em bactérias lácticas. A quantificação relativa do número de cópias do plasmídeo foi realizada por PCR em tempo real e como análise complementar utilizou-se a quantificação por absorvância a 260nm após purificação do plasmídeo. Embora haja alguma variação, parece existir um aumento do número de cópias do pTRKH3 em *L. lactis* LMG 19460 em 4 colónias. No entanto para outras colónias este aumento não foi tão evidente. A sequência modificada do RBS foi confirmada por sequenciação para os clones mais produtores e para os restantes. Finalmente, melhorou-se o rendimento de purificação (cerca de 3000%) dos plasmídeos adicionando 10mg/ml de lisozima durante o passo de lise celular.

Palavras chave: Bactérias ácido lácticas, vacinas de DNA, número de cópias de plasmídeo, Origens de Replicação, plasmídeo pTRKH3.

ABSTRACT

The vaccines based on plasmid DNA are a safer alternative to the traditional. In general plasmid vectors are produced more quickly and easier. *Escherichia coli* is the most common host strain for production of DNA vaccines. However, these gram-negative bacteria have lipopolysaccharides in the outer cell membrane, which acts as an endotoxin that binds to a complex of CD14/TLR4/MD2 receptors, activating the complement cascade resulting in a severe inflammatory reaction. This work aims to develop a safer alternative to DNA vaccines in Lactic Acid Bacteria since they are commonly used in the food industry, used as a probiotic and also for delivery of therapeutic proteins.

The process of growth at small scale has been optimized for strains *Lactococcus lactis* LMG 19460 and *Lactobacillus plantarum* CCUG 61730. It was also produced 1 liter in anaerobic conditions. Electrotransformation was achieved with NaCl method only for strain *L. lactis* LMG 19460. A modified plasmid DNA (pTRKH3) for vaccine with a change in *Ribosome binding site* (RBS) was produced, in order to increase the copy number of plasmid in Lactic Acid Bacteria. The relative quantification of plasmid copy number was performed by RT-PCR and by absorbance at 260nm after purification by miniprep as a complementary analysis. It was found in four clones of *L. lactis* LMG 19460 harbouring pTRKH3 an increased plasmid copy number. However, for other colonies this increase was not so evident. Cell disruption and purification process of plasmid DNA was obtained by alkaline lysis. It was achieved much higher amounts (3000%) of purified pDNA after addition of Lysozyme 10 mg / l to cell lysis.

Keywords: *Lactic acid bacteria*, DNA vaccines, plasmid copy number, origins of replication. Plasmid pTRKH3.

LIST OF CONTENTS

Agradecimientos / Acknowledgments.....	iii
Resumo	iv
Abstract	v
List of contents	vi
List of figures	viii
List of tables	ix
List of abbreviations	x
1. Background and goal.....	1
1.1 Specific purposes	1
2. State of the Art	1
2.1 Dna vaccines	1
2.2 Lipopolysaccharides	2
2.3 Lactic acid bacteria (LAB)	3
2.3.1 Applications in human health	4
2.3.2 Plasmids in LAB	5
2.4 Origins of Replication	5
2.4.1 Copy number control.....	5
2.4.2 Types of origin replication	6
2.4.3 Replicon pAM β 1	7
2.5 Ribosome Binding Site	8
3. Materials and Methods	9
3.1 Bacterial strains and plasmids.....	10
3.1.1 E. coli DH5 α	10
3.1.2 DH5 α Competent cells	10
3.1.3 Plasmid DNA pTRKH3	10
3.1.4 Transformation DH5 α with plasmid.....	11
3.1.5 Digestion pTRKH3.....	11
3.2 Lactic Acid Bacteria	12
3.2.1 Cell growth optimization	12
3.2.2 Competent cells.....	12
Method 1 – g-sgmsr:.....	12
Method 2 – 10mm Dithiothreitol (DTT) and 100mm Lithium Acetate (LiAc).....	13
Method 3 - 0.7 m Sodium Chloride (NaCl)	13
Method 4 - 8% Glycine.....	13
3.2.3 Electroporation of <i>L. lactis</i> LMG 19460 and <i>L. plantarum</i> CCUG 61730	13
3.3 Construction of pTRKH3 <i>in silico</i>	14

3.3.1	Site directed mutagenesis.....	14
3.3.2	Quantitative Real-time PCR assay	15
3.3.3	Colony PCR.....	16
4.	Plasmid primary isolation	16
4.1	Alkaline lysis	16
4.2	Concentration and Clarification	17
4.3	Hydrophobic interaction chromatography (HIC) in Äkta 100 purifier system	17
4.4	Diafiltration -Amicon®.....	18
5.	Results and Discussion	18
5.1	Optimization of the growth of LAB at small scale and bench scale.....	18
5.2	Constructions of sequences of RBS's.....	20
5.3	Optimization of LAB transformation	21
5.4	Quantification of relative plasmid copy number	23
5.5	Complementary method to quantify copy number of pDNA by Miniprep	26
5.6	Cell lyses and Chromatography	26
6.	Conclusion	29
7.	Future perspectives	29
8.	References.....	30
	Attachments.....	34
	Attachment 1 – TSS stock solution for DH5α competent cells.....	35
	Attachment 2 – MRS broth Panreac® Cultimed.....	35
	Attachment 3 - Others methods tested for Electroporation	35
	Attachment 4 - Primers for site directed mutagenesis.....	36
	Attachment 5 - PCR programe for site directed mutagenesis.....	36
	Attachment 6 – Assays LMG/pTRKH3 (wt)	36
	Attachment 7 - Assays LMG/pTRKH3-RBS 4	37
	Attachment 8 – Calibration Curve RT-PCR	38
	Attachment 9 - Agarose gel 1% - RT-PCR reaction	38
	Attachment 10 – Digestion of pTRKH3 with HIND III	39

LIST OF FIGURES

Figure 1 - General chemical structure of bacterial lipopolysaccharides	3
Figure 2 - Recent applications of LAB delivery systems	4
Figure 3 - ct-RNA based replication control.....	6
Figure 4 - Theta type replication	7
Figure 5 - Replicon pAM β 1	7
Figure 6 - Process overview - tasks and respective units operations	9
Figure 7 – Physical map of plasmid pTRKH3 and some restriction sites	11
Figure 8 - Growth curve of <i>L. lactis</i> LMG 19460 and <i>L. plantarum</i> CCUG 61730 harbouring or not pTRKH3.....	19
Figure 9 - Specific growth rate of <i>L. lactis</i> LMG 19460 and <i>L. plantarum</i> CCUG 61730.....	19
Figure 10 - Modification of RBS sequences in pTRKH3	20
Figure 11 - Estimated copy number of pTRKH3 for different colonies	24
Figure 12 - Estimated copy number for various assays	25
Figure 13 – A: HPLC Chromatogram of pDNA sample from the original purification procedure B: Agarose gel electrophoresis 1% of the eluted fractions from a purification procedure	27
Figure 14 – A: Chromatogram from a purification procedure using lysozyme. B: Agarose gel analysis of the eluted fractions from a purification procedure with lysozyme	28
Figure 16 – Confirmation in 1% agarose gel electrophoresis of plasmid pTRKH3 in RT-PCR reaction.....	38
Figure 15 - Standard curve for the relative quantification of plasmid copy number.	38
Figure 17 - Analysis of quality of pTRKH3 by 1% agarose gel electrophoresis	39

LIST OF TABLES

Table 1 - Examples of therapeutic molecules produced by recombinant LAB	5
Table 2 - Bacterial strains and plasmids.....	10
Table 3 - Cell number used in electrotransformation with NaCl method	14
Table 4 - Sequences RBS.....	15
Table 5 - Results of electrotransformation.....	22
Table 6 - Estimated number of copies of pTRKH3 by Miniprep.....	26
Table 7 - Electroporation conditions of method 1 - G-SGMRS	35
Table 8 - Electroporation conditions of method 2 – DTT/LiAc.....	35
Table 9 - Electroporation conditions of method 4 – 8% Glycine	36
Table 10 - Primers for site directed mutagenesis	36
Table 11 - Assays for RT-PCR - Overview of all cells banks LMG/pTRKH3 wt	36
Table 12 - Assays for RT-PCR - Overview of all cells banks LMG/pTRKH3 RBS 4	37

LIST OF ABBREVIATIONS

- bp** – Base pair
- C_t** - Threshold cycle
- Ct-RNA** – Countertranscribed Ribonucleic acid
- DNA** - Deoxyribonucleic acid
- DTT** – Dithiothreitol
- GIT** – Gastrointestinal tract
- G-SGMRS** – culture medium of competent cells containing Sucrose, Glycine, Glucose and Man Rogosa Sharp.
- GRAS** - Generally recognized as safe
- HIC** - Hydrophobic Interaction Chromatography
- LAB** – Lactic Acid Bacteria
- LB** – Luria Bertani
- LiAc** - Lithium acetate
- LPS** – Lypopolysaccharide
- MCS** - multiple cloning site
- MRS** – Man Rogosa Sharp
- mRNA** - Messenger ribonucleic acid
- dNTP** - deoxynucleotide triphosphate
- EDTA** - ethylene diamine tetra acetic acid
- oc** – Open circular
- O.D._{600nm}** – Optical Density read at 600nm
- pDNA** – plasmid DNA
- PCR** - polymerase chain reaction
- RNA** – Ribonucleic acid
- RT – PCR** - Real time - polymerase chain reaction
- RC** – Rolling circle
- sc** - Supercoiled
- U.V** – Ultraviolet

1. BACKGROUND AND GOAL

The goal of this work is to produce plasmid DNA (pDNA) by Lactic acid bacteria (LAB), to use as DNA vaccines.

Vaccines based on plasmid DNA are very promising tools both for the prevention of infectious diseases and for the treatment of acquired diseases. These vaccines have several advantages over traditional vaccines, namely the flexible genetic design, lack of infection risk and low cost of production in a microbial host [1]. These DNA vaccines are currently produced as plasmids in *Escherichia coli*. In this work, we propose an alternative GRAS (generally recognized as safe) host cell system for pDNA production: lactic acid bacteria. The established procedures for plasmid production and downstream processing were based in *Escherichia coli* [2] with some point modifications for LAB.

E. coli contains highly immunogenic lipopolysaccharides (LPS) that may be co-purified with pDNA [1]. In alternative, species of *Lactococcus* and *Lactobacillus* genera for pDNA production will be used [3] [4]. Lactic acid bacteria are widely used for the manufacturing of dairy products, being considered GRAS due to its non-invasive and non-pathogenic nature [5]. A major problem while using LAB for pDNA production is the low plasmid copy number which leads to low plasmid yields. In order to overcome this problem an increase in the number of copies of plasmid per cell will be developed in this work.

1.1 SPECIFIC PURPOSES:

- Production of a higher copy number of an engineered plasmid DNA, thus increasing the yield in the production of therapeutic molecules.
- Optimization of the cell growth of LAB.
- Optimization of electrocompetent LAB cells.
- Adaptation of plasmid extraction, separation and purification, considering that LAB are quite different from *E. coli*.

2. STATE OF THE ART

2.1 DNA VACCINES

Vaccines based on plasmid DNA are an alternative for the prevention of infectious diseases (e.g. malaria, tuberculosis, HIV, and others) and the treatment of some autoimmune and cancer diseases [2]. This is only possible due to the ability to induce both humoral and cellular immune responses against antigens encoded by recombinant DNA. The administration of plasmid DNA provides an immune response, including the activation of

CD8⁺ and CD4⁺ T lymphocytes, which secrete cytokines and have a regulatory role in antibody production [6].

Bacterial plasmid vectors used as DNA vaccines are developed for expression of proteins in mammals cells. If compared to virus vector, plasmids vectors have higher safety, lower cost and are easy to produce [7]. There are some essential elements in the plasmids used as DNA vaccines, as a promoter and a polyadenylation signal (polyA) for expression in mammalian cells, a selection marker, a prokaryotic replication origin for plasmid amplification in bacteria [7]. The ColE1 bacterial origin of replication found in numerous plasmids pUC series derives from *Escherichia coli*. This origin of replication provides a high plasmid copy number, which allows getting larger amounts of purified plasmids.

The DNA vaccine is taken by Antigen-presenting cell (APC) or others tissue resident cells such as muscle cells or stromal cells. The DNA endocytosed by cells remains in the cell nucleus without being inserted into the host cell genome [6]. Several encoded antigens may be secreted into the extracellular space, and can stimulate B lymphocytes to produce specific antibodies as being endocytosed by other APCs. They produced cytokines such as IL-12 and as IFN- γ which may act on the target cell which carries the transgene expression. The IFN- γ is an important cytokine in modulating of B cells to secrete IgG2a (a specific antigen, present in many infections by microorganisms) [6] [8] .

E. coli is commonly used for the production of DNA vaccines, since it is an organism that it's easy to grow at high densities [2], has a variety of cloning vectors and it's physiology and genetic is well known. On the other hand, it has a high content of endotoxins like a LPS.

Thus arises, the need for safer host producers, such as LAB. Currently some studies have been made using these organisms for heterologous expression of proteins and as vehicles for delivery of DNA [5] [9] [10].

2.2 LIPOPOLYSACCHARIDES

The bacterial LPS are a component of outer membrane of the gram-negative bacteria, have a molecular mass of 10-20 kDa, and protect the bacterium against the action of bile salts and lipophilic antibiotics [11].

LPS are located in the exoplasmic leaflet of the outer membrane, acts as an endotoxin and are strong stimulators of the innate immunity response of a mammalian host, by acting as a potent activator of macrophages which induce them to release inflammatory cytokines such as, tumor necrosis factor (TNF) and interleukin (IL-1, IL-6 and IL-8). Excessive production of cytokines in response to LPS is regarded as the cause of septic shock in humans and also the failure of many organs until the patient's death [12] [13].

In general, the LPS is a large molecule constituent of an amphipathic lipid (lipid A) covalently linked to a hydrophilic polysaccharide [14].

The LPS structure (as shown in figure 1) represents the main surface antigen, is composed of an outer or distal segment, an O-chain or O-antigen, an outer core segment, an inner core segment, and finally, the medial lipid A portion [13] [15]. It has been proven, in previous studies, that the lipid A is the endotoxic centre of the molecule [12] [14].

The O-antigen is composed by a sequence of repeating units of oligosaccharide, specific to each bacterial species and strains. The core region is an oligosaccharide containing characteristic sugar residues,

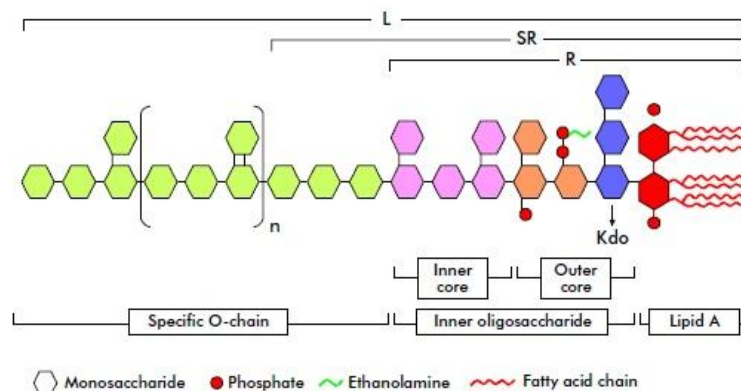


Figure 1 - **General chemical structure of bacterial lipopolysaccharides:** The portion Rough type LPS (R) do not contain specific O-chain, semi-rough type (SR) just contain a repetitive unit of specific O-chain, and smooth type (S) contain two or more repetitive units of specific O-chain [15].

Kdo and heptose. However, chemical variation is more limited than that of O-antigen [16]. The *E. coli* type lipid A consists of one glucosamine disaccharide, two phosphates and six acyl groups [14] and shows a strong immuno-stimulating response [16].

What happens is Toll-like receptor 4 (TLR) in association with MD-2 is responsible for the recognition of LPS. The TLR is a type I transmembrane protein expressed on immune cells. This is a major signaling receptor of the innate immune system and recognizes a wide variety of microbial products. The LPS is transferred to TLR4-MD-2 by two accessory proteins, LPS-binding protein and CD14 [11] [14]. Thus, it activates the signaling cascade leading to the synthesis of inflammatory mediators, like cytokines. Thus, it can be differentially stimulated two pathways inflammatory responses. The myeloid differentiation primary response protein 88 (MYD88), leads to the production of pro-inflammatory cytokines. Whereas the less inflammatory TRIF pathway which stimulates the expression of interferon β (IFN β) and inducible genes that are important for adjuvanticity [11].

2.3 LACTIC ACID BACTERIA (LAB)

Lactic acid bacteria (LAB) are a group of non-pathogenic and non-invasive gram-positive bacteria, which occupy different ecological niches such as digestive tract of human and animals [5] [17]. There are several genera of LAB, classified by their morphological and physiological characteristics, include species of *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* [18]. Species of lactobacilli and streptococci are highly represented in different mucosal compartments of the human body [18]. These bacteria, which produce

lactic acid as major product, are used in numerous fermentations processes of animal origin, and can be used either alone or together with other organisms [17].

In the last decades, LAB have demonstrated high interest as mucosal delivery vehicles. This follows from to achieve efficient strategies to deliver vaccine antigens, microbicides and therapeutics to the mucosal tissues [18]. It is considered as probiotics once “*Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host*” [18]. Furthermore, provides some benefits as probiotics in human health, such as detoxifying carcinogens, enhancing immune response, metabolizing cholesterol, and others [19]. LAB are good candidates for the development of oral vectors, because some strains play an important role in maintaining intestinal homeostasis and exert beneficial effects. They have the capacity to survive the passage through the gastrointestinal tract (GIT) and do it so makes these bacteria excellent candidates for mucosal targeting of therapeutic molecules [10]. In figure 2 summarizes the current applications of LAB, and the molecules which have been produced.

Lactobacillus genus can remain viable for a while in GIT. For example some strains of *Lactobacillus plantarum*, have probiotic effects with anti-inflammatory properties [20].

Also they can grow in the presence and absence of oxygen. *Lactobacillus plantarum* secretes antimicrobial compounds that inhibit pathogenic gram-positive and gram-negative colonies from forming [21]. *Lactobacillus* strains have a general beneficial effect and thus can be used for therapeutic purposes [22].

2.3.1 APPLICATIONS IN HUMAN HEALTH:

The capability to be administered by the mucosal way represents a significant advantage of the use of *L. lactis* based vectors. Mucosal surfaces (oral, intranasal, vaginal, or rectal) represent an important gateway for many pathogens [23]. In the table 1 shows some application by LAB and their benefits in human health.















Product	Model	Application
DNA 		Vaccines
Peptide 		HIV-1, fusion inhibitors; colitis, trefoil peptides
ScFvs 		Anti-infectives
Antigens 		Vaccines
Cytokines 		Inflammatory bowel disease and colitis, vaccine adjuvants
Enzymes 		Therapy for colitis and enzyme deficiencies
Allergen 		Therapy and prevention of allergy

Figure 2 - **Recent applications of LAB delivery systems:** The single-chain variable fragments (scFvs) molecule, could be used for different applications (therapy, anti-infectives or passive immunization). Not all of these potential applications have been published in the literature. [18]

Table 1 - Examples of therapeutic molecules produced by recombinant LAB

Applicability in Disease	Beneficial effects
Inflammatory Bowel Disease	Includes Crohn's disease and ulcerative colitis. Recombinants LAB produce anti-inflammatory molecules, such as IL-10. Thus, their direct delivery to the intestinal mucosal and preventing their degradation by the high level proteolytic environment encountered in the GIT [22] [23].
Anti-Tumoral	For example, in a colon rectal cancer, the LAB may reduce the risk by competitive exclusion of pathogenic bacteria involved in carcinogenesis. It also reduces the activity of procarcinogenic enzymes, such as β -glucuronidase [24].
Allergy	<i>L. lactis</i> vectors showed anti-allergic properties for food allergens in mouse as an animal model [23].
Anti-Viral	The Interferon-omega (IFN- ω) produced by <i>L. lactis</i> is effective on enteric viral infections [23].
Body Weight and Obesity	The engineered <i>L. lactis</i> expressing epidermal growth factor (EGF) was proposed to have applications in dietary supplements for children during weaning when intestinal development is most required [23].

2.3.2 PLASMIDS IN LAB:

Several lactic acid bacteria harbor *cryptic* plasmids (1.5 to >30 kb in size), which potentially can serve as vectors [25] [26]. Although most lactobacilli plasmids are smaller than 10kb, is known that some plasmids can be larger until 150kb [19].

A few known plasmids may encode important traits such as resistance to phages or antibiotics, lactose catabolism, production of proteolytic enzymes and encoding different bacteriocins [25] [27]. Many of these plasmids are not yet characterized and future may bring new information to other molecules which may also be engineered for the development of cloning and expression vectors.

Several *Lactobacillus* species often harbor a number of these plasmids and DNA sequence analysis has revealed that they replicate a rolling circle replication (RCR) mechanism, and also theta mechanism [25] [26].

2.4 ORIGINS OF REPLICATION:

2.4.1 COPY NUMBER CONTROL

Bacterial plasmids replicate autonomously and may bring benefits to the host, such as drug resistance or metabolic pathways. Several resistant natural plasmids have been report to carry virulence factors, such as bacteriocins, siderophores, cytotoxins, or adhesion factors and virulence plasmids have been described to carry resistance genes [28].

The origin of replication determines the plasmid copy number, and their stability is affected by copy number. A positive effect of a high copy number is the greater stability of the plasmid. On the other hand, a high number of plasmid copies generally decrease the growth rate, thus possibly allowing for cells with few plasmids to dominate the culture, once

they grow faster. Plasmids that utilize the same replication system cannot co-exist in the same bacterial cell, may exist incompatibility [29].

Thus, there are two basic systems which control the copy number of the plasmid, depending on the type of negative control component used:

- directly repeated sequences (iterons) that complex with cognate replication (Rep) initiator proteins;
- antisense RNAs that hybridize to a complementary region of an essential RNA, therefore termed countertranscribed (ct) RNAs [29] [30].

In this last case the ctRNA binds to its complimentary RNA (necessary for plasmid replication), inhibiting (directly or indirectly) replication of the plasmid, (as shown in figure 3). This inhibitory ctRNA is synthesized at a higher rate than that of the essential RNA, although having a short half-life. This happens because the ctRNA is transcribed by a constitutive promoter, and its intracellular concentration is proportional to plasmid copy number [28] [29]. The ctRNAs can regulate plasmid copy number (either alone or with the help of proteins).

Thus, it may be involved in a variety of mechanisms including inhibition of primer maturation, inhibition of translation of the essential Rep protein, transcriptional attenuation, or inhibition of pseudo knot formation [29]. The interaction in ctRNA and mRNA promotes formation of the terminator and thus can lead to premature termination of Rep mRNA synthesis [31].

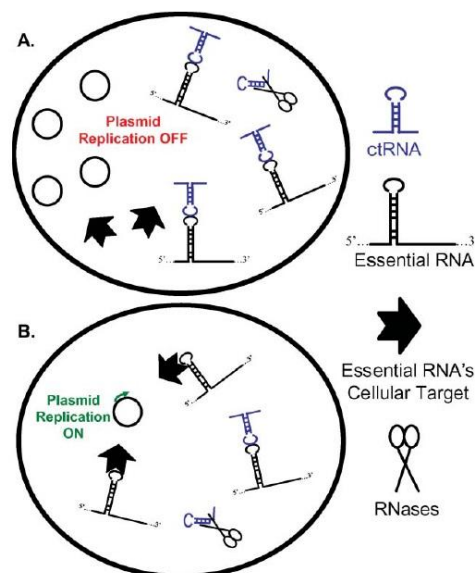


Figure 3 - ct-RNA based replication control [29]

2.4.2 TYPES OF ORIGIN REPLICATION

Plasmids DNA replicate using two modes, as RCR and theta mechanism. In most RCR plasmids, transcription of the initiator gene and leadingstrand replication proceed in the same direction. The RCR derived plasmids may not be suitable for the cloning of some genes, such as large genes or genes with high GC content, but they are generally high-copy-number plasmids (50 copies/cell) and usually contain a pSH71-based replicon (with *repC* and *repA* genes, from *Lactococcus lactis*) [3] [4], which encodes a replication initiation protein [19]. To overcome this problem it can be used theta replicating plasmids, which are structurally and segregationally more stable than RCR plasmids, and can hold larger DNA inserts [5] [32].

For example, pAM β 1 replicon based plasmids that contain regulated *rep* genes [33]. In a study, the *spac-I* promoter upstream of ORF E was inserted and this plasmid pAM β 1spac replicated efficiently resulting in about 100 plasmids copies/cell in *Bacillus subtilis* [34].

Three classes of theta replicating plasmids are known and designated A, B, and C. Class A includes plasmids which encode a replication protein (Rep) and have a characteristic replication origin, designated ori A [34]. The figure 4 represents the theta mechanism of replication. The origin of replication has a unique nucleotide sequence necessary for replication of the DNA molecule. DNA replication is initiated at the origin (ORI) and continues in both directions around the circular molecule, as the growth forks move merge and two daughter molecules are produced [35]. For cloning in *Lactococcus lactis*, several studies have shown that the theta type plasmid have more stable segregation then the RCR type plasmid [36].

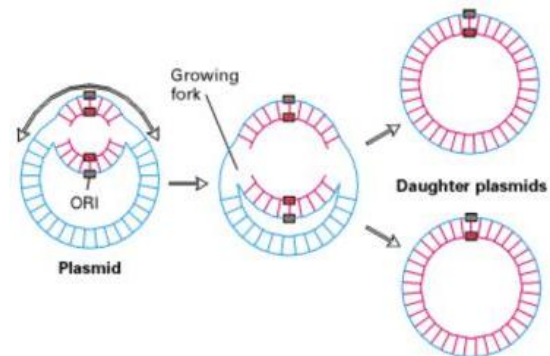


Figure 4 - Theta type replication [35]

2.4.3 REPLICON pAM β 1

The pAM β 1 plasmid was initially isolated from *Enterococcus faecalis*, is a low copy number and the replication is of unidirectional theta [37]. A lot of plasmids that were isolated from *streptococci* and *enterococci* have much similarity with pAM β 1 replicon. This initial mechanism involved Rep protein, the origin and the host encoded DNA polymerase I [31]. The initiation of leading strand synthesis occurs within a -44 bp origin located immediately downstream of the Rep gene [31].

A few elements are involved in this control, and are located within a -1 kb segment mapping upstream of the RepE open reading frame (ORF). The *repE* gene coding for a replication protein of 57-kDa [34]. This region is responsible for the copy-control, as shown in Figure 5. The synthesis of RepE is strongly controlled, because it is rate limiting for replication. It contains two ORFs, D and F, located on the same strand as RepE, and an intergenic region of -350 bp carrying two major promoters (P_{DE} and P_{CT}) and two rho-independent transcription terminators (T_{DE} and T_{CT}) [31] [37].

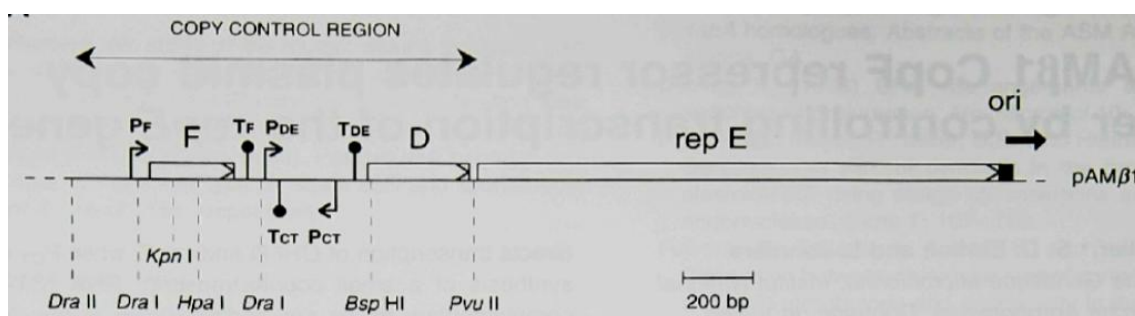


Figure 5 - Replicon pAM β 1 [37]

It was shown on earlier studies that the P_{DE} promoter is required for transcription of the ORF D and REP E gene. However, RepD is not necessary for replication and its function remains unknown [38]. Nevertheless, the P_{CT} directs synthesis of a small countertranscript RNA (CT-RNA), complementary to the non-coding region of the ORF D repE mRNA [31] [37]. The terminator (T_{DE}) inhibits transcription initiated in the P_{DE} , so, like the other terminator (T_{CT}) inhibits transcription of the P_{CT} [37].

Based on previous experiments, one can confirm that, T_{DE} is active and is a key element in the negative regulation of P_{DE} directed transcription [37] [31].

In previous studies it was verified that CopF negatively regulates pAM β 1 replication by binding to an operator which is located immediately downstream of the *copF* gene [37]. Thus, the CopF acts as a repressor of the RepE promoter. To test that CopF negatively regulates pAM β 1 copy number, by repressing RepE transcription, a few experiments were done and was carried out a deletion a upstream *KpnI* restriction site. So, as consequence enabled a slight increase of copies of plasmid [37]. It was also observed which mutations in the P_{CT} promoter or in the transcription terminator (T_{CT}), had no effect on CopF mediated regulation and did not affect the activity of the promoter and terminator of the countertranscript (P_{CT} and T_{CT}) [31]. These experiments showed that CopF acts independently of the attenuation system and they also observed that, CopF mediated regulation, does not require ORF D. Thus, the countertranscript-driven attenuation mechanism in conjunction with CopF regulatory system repress 100-fold the transcription of the *repE* gene [31].

2.5 RIBOSOME BINDING SITE

Every bacterial mRNA has a ribosome binding site (RBS), for producing polypeptide product. The RBS is a sequence upstream of the start codon where the ribosome binds to the mRNA to begin its the translation of the mRNA into a protein [39]. In prokaryotic cells the ribosome binding site is denominated Shine Dalgarno sequence (SD) [40].

Although transcript properties such as mRNA stability and codon usage are known to influence microbial translation, initiation is often the rate-limiting step in translation [41]. It has been verified the distance between the SD sequence and the initiation codon affects the translation [40]. It have been identified a number of sequence specific with characteristics that can decrease translation initiation rates, and therefore result in low expression levels [42]. The presence of secondary structure in the 5' region of the mRNA can limit translation due to sequestration of the 16S hybridization region (SD) sequence or the start codon [43]. Secondary structures that limit translation efficiency may be present in the 5' untranslated region of the transcript as well as in the coding region downstream of the initiation codon [43]. However, it is known that, gram positive bacteria differ in some characteristics of their

translational machinery in comparison to gram negative. Thus, a predicted strong RBS can have different effects depending on the organism.

The change of the original RBS in pAM β 1 origin replication, might lead to a higher production of RepE protein and therefore more copies of pDNA.

3. MATERIALS AND METHODS

The strains *Lactococcus lactis* (LMG 19460) and *Lactobacillus plantarum* (CCUG 61730), which were chosen for this study are both natural inhabitants of the gastrointestinal tract [17] [44] and also good candidates as delivery vehicles.

Although these advantages, gene manipulation and vector amplification are difficult in LAB. Therefore *Escherichia coli* (strain DH5 α) was selected to perform these techniques, using a shuttle vector for LAB and *E.coli*. The shuttle vector contains the replicons and the genetic markers functional in LAB and *E.coli*.

The following diagram (figure 6) illustrates all phases of the work. The techniques used have been optimized taking in account the characteristics of the LAB.

All materials and solutions used were previously sterilized by autoclave at 121°C for 20 min, and bacterial cells always manipulated in sterile conditions in a laminar flow chamber.

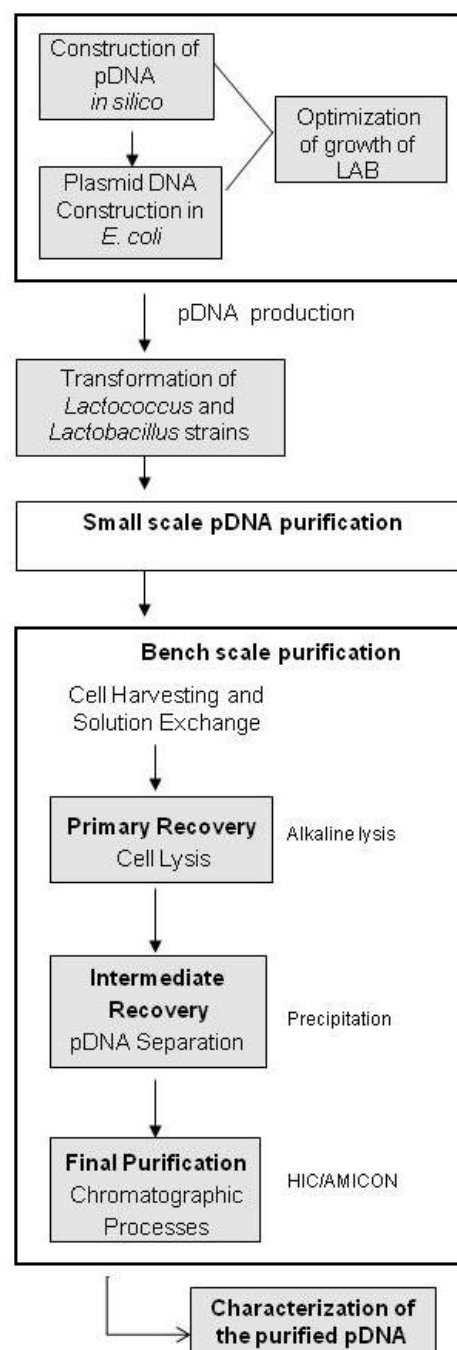


Figure 6 - Process overview - tasks and respective units operations

3.1 BACTERIAL STRAINS AND PLASMIDS:

The bacterial strains and plasmids used in this work are show in Table 2.

Table 2 - Bacterial strains and plasmids

Strain or plasmid	Replicon	Characteristics	Source
<i>E. coli</i> DH5 α		<i>F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG lacZΔM15 hsdR17</i>	Invitrogen
<i>Lactococcus lactis</i> (LMG 19460)		Wild type, plasmid free	BCCM Collection
<i>Lactobacillus plantarum</i> (CCUG 61730)		Wild type, plasmid free	Collection of Culture University of Göteborg
pTRKH3	p15A ori, pAM β 1 ori	Tet ^R , Ery ^R	BCCM/LMBP collection, University of Ghent, Belgium

3.1.1 *E. coli*/DH5 α :

E. coli DH5 α cells were grown in a pre-inoculum overnight at 37°C in 15ml falcon tubes containing 5ml of *Luria Bertani* (LB) medium (20g/L) from Sigma Aldrich supplemented with 10 μ g/ml of tetracycline stock solution (10mg/mL) when containing pTRKH3 vector or its derivatives, at 37°C with 250 rpm in an ARALAB orbital shaker model AGITORB 200. When cells reached the exponential growth phase they were transferred to 100ml Erlenmeyer flask containing 20ml of LB medium and incubated at 37°C and 250 rpm with 20 μ l of the same tetracycline stock solution, for 4-5 hours or until they reach the O.D._{600nm} of 1-1.5 measured in U-2000 spectrophotometer (Hitachi®). Then the cells were used to make cell banks in 20% (v/v) glycerol, the 100 μ l aliquots were stored at - 80°C in 1.5ml microcentrifuge tubes.

3.1.2 DH5 α COMPETENT CELLS:

The cells were grown up to the O.D._{600nm} 1-1.5 (as described in section 3.1.1) and were centrifuged at 4000g for 10min. Supernatants were discarded, and the pellets were resuspended in 2ml of filtered TSS buffer (attachment1) and incubated for 10min on ice. Then aliquots with 100 μ l were prepared and stored at - 80°C in 1.5ml microcentrifuge tubes.

3.1.3 PLASMID DNA pTRKH3:

The backbone plasmid used in this work was the pTRKH3 (figure 7) which is a shuttle cloning vector for *E. coli*, *Lactococcus*, *Enterococcus*, *Streptococcus* and *Lactobacillus*. The replicon includes an *E. coli* p15A origin as medium copy number (30-40 copies of plasmid per cell) and an *Enterococcus faecalis* pAM β 1 origin, denominated high copy number (45-85 copies in Gram-positive strains). The tetracycline resistance is only expressed in *E. coli* (which can be insertionally inactivated, having several sites of cloning), while the erythromycin resistance is expressed both in *E. coli* and in Gram-positive bacteria. It was

demonstrated in previous studies [45] [46], that cloning vector pTRKH3 can be transmissible to certain strains of LAB. Through, the direct cloning in LAB, the need arises to use an intermediate host *E. coli*.

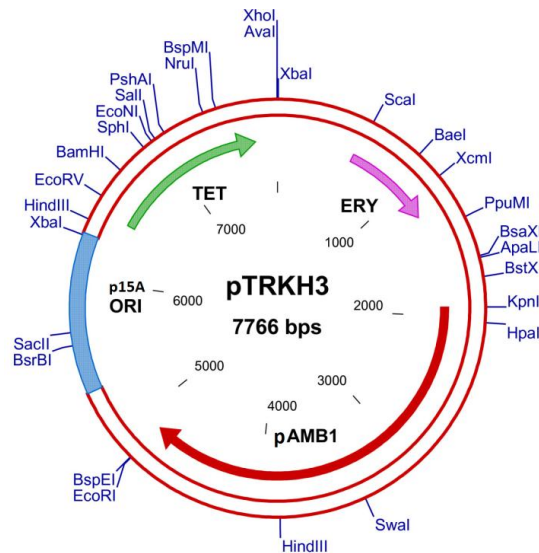


Figure 7 – Physical map of plasmid pTRKH3 and some restriction sites [46]

3.1.4 TRANSFORMATION DH5 α WITH PLASMID.

DH5 α cells were transformed with the plasmid pTRKH3, by heat shock. This was performed by the addition of 40 μ l of the plasmid (if is mutated or 10 μ l if is original pDNA) to the competent cells, for 30min on ice. The mixture was heated for 1min 30s at 42°C and then chilled on ice for 2min followed by the addition of 1ml LB and incubation at 37°C. Finally, cells were plated on selective LB agar medium supplemented with 10 μ g/ml, tetracycline and incubated at 37°C, approximately for 24 hours. Subsequently, were made cell banks as described in section 3.1.1. Plasmid DNA was purified with High Pure Plasmid Isolation Kit (Roche) following manufacturer's instructions. The pDNA obtained were eluted in 100 μ l elution buffer and stored at 4°C.

3.1.5 DIGESTION pTRKH3

In order to confirm the quality of the pDNA a digestion with Hind III restriction enzyme (restriction site A[^]AGCTT) (Promega) which cuts plasmid at two sites and produces two fragments 5265pb and 2501pb. Each digestion procedure consisted of 500ng of pDNA, 0.5 μ l of enzyme (10U/ μ l), 10 % of 10X Buffer B and filtered sterilized Milli-Q water to a determined final volume (10-15 μ l). All mixtures were incubated for 2h at 37°C. After that a 1% agarose gel electrophoresis was run.

3.2 LACTIC ACID BACTERIA

3.2.1 CELL GROWTH OPTIMIZATION:

The LAB cells were anaerobically cultured on Man Rogosa and Sharp (MRS) medium at 37°C in case *L. plantarum* CCUG 61730 and at 30°C for *L. lactis* LMG 19460 with gentle agitation at 100 rpm. LAB cells were grown in a pre-inoculum overnight at 37°C in 15ml falcon tubes containing 15ml of MRS medium (52g/L) from Panreac Cultimed (see attachment 2 for the composition) supplemented with 5µg/ml of erythromycin stock solution (150mg/ml), when containing the pTRKH3 vector, in an ARALAB orbital shaker model AGITORB 200.

The next day, the pre inoculum was used to start a new culture, by the addition of these cells to a fresh medium up to O.D._{600nm}=0.1. The cells grew for additional 6 to 7 hours, until the end of exponential phase, and cell banks were made in 20% (v/v) glycerol. The 100µl aliquots were stored at - 80°C. The LAB cells cultures were tested small scale (cell growth in 15ml falcon tubes containing 15ml of MRS medium) and at bench scale (cell growth in 500ml Erlenmeyer flask containing 300ml MRS medium). To perform alkaline lyses, the cells were grown in a 2 liters Erlenmeyer flask containing 1 liter of MRS medium. In order to characterize the growth of *L. lactis* LMG 19460 and *L. plantarum* CCUG 61730 at small scale and bench scale growth curves were determined. For this, the growth was initiated at O.D._{600nm}=0.1 in both cases, and from that, the O.D._{600nm} was measured every hour.

3.2.2 COMPETENT CELLS

This procedure was done with strains without native pDNA, to avoid some incompatibility. For the transformation of LAB we tested some protocols for making cells competent as described below.

Method 1 – G-SGMRS:

This protocol was adapted from [47]. The LAB cells were grown overnight at 30°C and 37°C, depending on the strain, in 15ml falcon tubes containing 15ml of MRS medium and 0.5M sucrose, 2.5% glycine, and 0.5% glucose. The growth started with O.D._{600nm}=0.1, in an 100ml Erlenmeyer flask containing 100ml of the same new fresh medium.

The cells were grown until early and middle of exponential phase and the growth was stopped in ice and then cells were centrifuged for 10min at 4000g. Then, the cells were washed with 50ml of ice-cold wash solution (0.5M sucrose, 10% glycerol), and centrifuged for 15min at 6.000g, at 4°C. Subsequently, the cells were washed twice. First, the cells were kept on ice for 15min in 25ml of solution containing 0.5M sucrose, 10% glycerol, and 0.05M EDTA and centrifuged for 15min at 6.000g, at 4°C. Then, cells were washed with 12.5ml of ice-cold wash solution and centrifuged for 20min at 6.000g, at 4°C. Finally cells were

resuspended in 0.5ml of ice cold wash solution. Aliquots of 100 µl were made and cells were stored at – 80°C.

Method 2 – 10mM Dithiothreitol (DTT) and 100mM Lithium acetate (LiAc)

This protocol was done according to a modification of [46]. The LAB cells were growth overnight (as described in method 1) only with MRS medium. The new growth started with O.D._{600nm}=0.1 in a 15ml falcon tube containing 15ml of a same new fresh medium. After reaching the corresponding O.D._{600nm} (see attachment 3, table 9 with the conditions) the LAB cells were harvested in order to have 10⁹ cells and centrifuged. The cells pellet following the pretreatment with LiAc and DTT was as follows: 10⁹ cells were suspended at room temperature for 30min in 8ml of 100mM LiAc, 10mM DTT, 0.6M sucrose, and 10mM Tris-HCl, pH 7.5. Following treatment, the cells were centrifuged for 10min at 4000g, at 4°C and washed for 4 times with ice cold Milli-Q water and following the electroporation. In this method, electroporation was performed with fresh cells and was not made cell banks.

Method 3 - 0.7 M Sodium Chloride (NaCl)

This protocol was adapted from [48]. The LAB cells were grown overnight as described in method 1 only with MRS medium. The new growth started with O.D._{600nm}=0.1, in a 100ml of Erlenmeyer flask containing 100ml of a same new fresh medium supplemented with 0.7M NaCl. The cells were grown until early and middle of exponential phase, the growth was stopped on ice and then was centrifuged for 10min at 4000g, at 4°C. Thereafter, the cells were washed for 4 times with ice cold Milli-Q water and aliquots of 100µl were made with 20% glycerol which were stored at – 80°C. In table 3 (next section) is shown the O.D._{600nm} they reached and the cell number used for electroporation producing transformants.

Method 4 - 8% Glycine

This protocol was adapted from [49] [50] [51]. The *L. plantarum* CCUG 61730 cells following the same procedure of Method 3 NaCl. By medium of the competent cells was added 8% glycine. The *L. plantarum* CCUG 61730 were harvested (see attachment 3 and table 10) and centrifuged at the same conditions. The cells were washed for four times with ice cold Milli-Q water and cells bank was made at the same conditions.

3.2.3 ELECTROPORATION OF *L. LACTIS* LMG 19460 AND *L. PLANTARUM* CCUG 61730

Electroporation was optimized according to [46] [47] [51]. The electroporation cuvettes were maintained 30min on ice before electroporation. The cell number was calculated according to Jones, S., *et al* [52], [53] (O.D._{600nm} = 0.1 or 7 x 10⁷ CFU). The number of cells, the amount of pDNA and electrical pulse used in the methods (G-SGMRS, DTT / LiAc and 8% Glycine) are shown with more detail in attachment 3. Here is described the conditions in

the NaCl method produced *L. Lactis* LMG 19460 transformants. In the NaCl method, electroporation was performed taking account the number of cells (as shown in table 3) using an electroporator with pulse controller (BTX ECM 399). In some cases, was required add 2 or 4 frozen aliquots and centrifuge the cells in order to be concentrated in a volume of 100µl. Electroporation was performed by a single pulse at 2-500V, 200 Ω, and 25 µF (pulse length ranged of 4.6 ms to 4.8 ms), in 2 mm electroporation cuvettes, using 1000ng of purified plasmid DNA. The cell suspension was diluted immediately by the addition of 1ml MRS and incubated for 3 hours at 30°C or 37°C (depending on the strain) without agitation. After the expression period, cells were plated onto MRS agar 2% supplemented with erythromycin (5 µg/ml), and incubated for 72h for selection of transformants. Appropriate controls were made omitting pDNA or the electric pulse. Transformation was confirmed then by colony PCR. Cell banks were made and pDNA purification was done by nucleospin plasmid (Macherey Nagel Bioanalysis). The plasmid extracted was digested with Hind III (as shown in section 3.1.5).

Table 3 - Cell number used in electrotransformation with NaCl method

Strains	O.D. 600nm	Cell concentration in frozen aliquots	Number of cells used in electroporation	Variation in electric pulse
<i>L. lactis</i> LMG 19460	0.63	2.94 x 10 ⁹ cells in 100 µl	5.89 x 10 ⁹ CFU and 1.17 x 10 ¹⁰ CFU	2 -500 V
	0.43	2 x 10 ⁹ cells in 100 µl	4 x 10 ⁹ CFU and 8 x 10 ⁹ CFU	2-500V
<i>L. plantarum</i> CCUG 61730	2.16	9.97 x 10 ⁹ cells in 100 µl	9.97 x 10 ⁹ CFU and 1.994 x 10 ¹⁰ CFU	2-500 V and 2x 2-500 V
	2.16	9.97 x 10 ⁹ cells in 100 µl	9.97 x 10 ⁹ CFU and 1.994 x 10 ¹⁰ CFU	2x 2-500 V and 3x 2-500 V

3.3 CONSTRUCTION OF pTRKH3 *IN SILICO*

In order to construct the ribosome binding site (RBS) it was used the *ApE® software*.

The RBS is a sequence found in prokaryotic mRNAs and its denominated Shine Dalgarno sequence. The Ribosome binds to the RBS and begins the translation at the start codon (ATG). The choice of RBS will affect protein expression levels. In order to produce an increased number of plasmid copies, it was tested two different RBS using the *Anderson RBS library* [39]. Thus far, the RBS library has not been experimentally characterized. The Anderson library was used to predict and design a two putatively stronger RBS sequences to replace the wild-type present in pAMβ1 origin of pTRKH3.

3.3.1 SITE DIRECTED MUTAGENESIS

For site directed mutagenesis it is necessary, that the mutagenic primer has high homology to the DNA strand which will be synthesized. The PCR Primers were designed using *QuikChange Primer Design* [54], and the sequences are in the attachment 4. Table 4 shows the strategy adopted in the construction of primers to switch the RBS.

Table 4 - Sequences RBS

Original RBS sequence	GTG GA GTT
RBS 4	
One pair of primers to switch 4 nucleotides	GGG GA CAA
RBS 5	
One pair of primers to switch 3 nucleotides	CAG GA CCC
One pair of primers to switch 2 nucleotides	CAG GA CCC

The commercial kit Novagen® KOD Hot Start DNA Polymerase and the Thermo Hybaid Px2 thermocycler were used for the site directed mutagenesis. Each reaction mixture of 50µl contains 50ng of template pDNA, 1µl of KOD HotStart DNA polymerase (1U/µl), 4.5µl MgSO₄ (25mM stock concentration), 5µl Buffer KOD (10X concentrated), 10µl dNTP's (2mM stock concentration), 10 pmol each of forward and reverse primers and PCR grade water to obtain the final volume. The program used is shown in attachment 5. At the end of the PCR reaction was added to the mixture 1µl of the enzyme DpnI. The template DNA is methylated, and thus susceptible to DpnI digestion. The modified plasmids were used to transform to DH5α cells, where plasmid nicks are repaired and amplification occurs. Plasmid DNA proceeded to be purified with High Pure Plasmid Isolation Kit (Roche). To confirm the plasmid quality a digestion was made with Hind III restriction enzyme (as shown in section 3.1.5). The fragments obtained were run in a gel with 1% of agarose. The confirmation of the mutagenesis was made by sequencing by *Stabvida*.

3.3.2 QUANTITATIVE REAL-TIME PCR ASSAY

The quantification of plasmid copy number in *L. lactis* LMG 19460 was performed with the Roche LightCycler™ detection system using the FastStart DNA Master SYBR Green I kit™. The detection of pDNA was performed by amplification of a 190 bp sequence from the *erm* gene, the selective mark for erythromycin resistance (Forward primer: 5' - CCA TGC GTC TGA CAT CTA TCT G - 3' Reverse primer: 5' – CTG TGG TAT GGC GGG TAA GT – 3'). The *L. lactis* LMG 19460 cells necessary for RT-PCR were grown up to O.D._{600nm} = 1 as previously described. Cells were harvested, diluted in 100 µl and stored at -20°C. The same amounts of cells (5 x 10⁴) were used in all experiments including the pDNA standard curve. Plasmid DNA for standard curve was extracted from *E. coli* DH5α according to the Roche Miniprep Kit protocol. Serial five folds dilutions (1pg, 10pg, 100pg, 1000pg, 10000pg per capillary) of plasmid were conducted in triplicates to establish the standard curve. The C_T values were used to calculate a linear regression line by plotting the logarithm of template concentration (X-axis) versus the corresponding threshold cycle (Y-axis), (as shown in attachment 8).

Each 20µl of reaction mixture volume contained 2µl of the 10x SYBR Green mixture, 1µl of each primer (10µM final concentration), 1.6µl of MgCl₂ (3mM final concentration), 2µl of sample and the remaining volume was fulfilled by PCR grade water. Determination of pDNA

concentration in the *L. lactis* LMG 19460 cells was done in triplicate, for each analysis and performed by adding in each capillary was 2µl with 5×10^4 of these cells, 2µl of PCR grade water, and 16µl of the PCR reaction mixture. Reactions were incubated at 95°C for 10min to activate FastStart DNA polymerase and lyse cells, followed by the amplification step consisting of 30 cycles of 10s at 95°C, 5s at 56.9°C and 7s at 72°C, [55] reactions were kept at 70°C for 30s, while fluorescence signal was continually monitored, for melting curve analysis. The final cycle, was a cooling step at 40°C, 30s. Standard curves obtained in the reaction provide the relation between plasmid copy number and the C_T , a relative quantification method. The calculations to obtain the number of copies were based on the calibration curve, by finding the log of the concentration, thus, knowing the concentration of pDNA and its mass. Therefore, allowed knowing the number of moles and molecules. The number of copies is obtained by dividing the number of molecules for the number of cells in the reaction (5×10^4 cells).

3.3.3 COLONY PCR

A PCR colony analysis was done to confirm cell transformation with pTRKH3. The pDNA was amplified for *erm* gene. The transformed colonies were picked into an eppendorf tube contain 70µl the Milli-Q water autoclaved and filtered (filter 0.1 µm). The 50µl of this volume was incubated at 99°C for 5min to burst through the cells, the remaining 20µl were plated of positive PCR products. After the heat treatment, the cells were centrifuged at 11.000g for 1min and 10µl of the supernatant (containing pDNA) were used to add to PCR reaction. The negative control was performed with *L. lactis* LMG 19460 cells without pDNA.

The commercial kit used for amplification was NovaTaq™ Hot Start Master Mix kit – Novagen. Each reaction mixture of 50µl contains 10µl of pDNA, 25µl of Master Mix, 0.5µl (10µM stock concentration) each of forward and reverse primers and PCR grade water to obtain the final volume. The products of PCR reaction were run in a 1% agarose gel electrophoresis.

4. PLASMID PRIMARY ISOLATION

4.1 ALKALINE LYSIS

L. lactis LMG 19760 cells harboring pTRKH3 plasmid, previously stored at -80°C were cultivated overnight as described in section 3.2.1. The next day, these cells were used to inoculate a 100ml Erlenmeyer flask containing 100ml of fresh medium, with a starting O.D._{600nm} of 0.1. After 8 hours, these cells were transferred to a 2L Erlenmeyer flask with 1L of MRS medium, supplemented with 5µg/ml of erythromycin. These cells were grown overnight (for 12 hours) at 30°C and 100rpm until the early stationary phase, corresponding to an O.D._{600nm} = 1.20-1.40. In order to collect the cells, the culture was centrifuged at 6000g

for 15min at 4°C. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in buffer P1 (50mM glucose, 25mM Tris-HCl, 10mM EDTA, pH 8.0), using vortex. Filtrated stock of Lysozyme with concentration 10mg/ml was added to buffer P1. The required volumes of P1 were calculated with the previously measured O.D._{600nm} of the cell culture and following equation. The volume of P2 (0.2M NaOH, 1% (w/v) SDS) and P3 (5M potassium acetate, 6.8M glacial acetic acid) solutions, was the same volume of P1.

$$V_P = \frac{O.D_{600nm} \times V_{culture}}{60} \quad \text{Equation 1}$$

Where V_p is the volume of “P” solutions and $V_{culture}$ is the volume of culture to be used. Alkaline lysis was performed by adding the P2 solution, followed by an incubation of 10min at room temperature. The P3 solution was added followed by gentle homogenization. To precipitate genomic DNA, the mixture was left on ice for 10min. The lysate was centrifuged at 20.000g for 30min, at 4°C, in a Sorvall RC6 centrifuge with a SS-34 rotor, in order to remove genomic DNA, proteins and cell debris. The supernatant was placed in new tubes and centrifuged with the same settings as before to ensure complete removal of cell debris.

4.2 CONCENTRATION AND CLARIFICATION

In order to precipitate pDNA a previously calculated volume (70% volume of the lysate volume) of isopropanol (100% v/v) was added to the lysate, followed by careful and gentle homogenization and a static incubation for 2 hours at -20°C for precipitation. The mixture was centrifuged for 30 min at 4°C at 20.000g and the supernatant was discharged. The pellets were left to dry overnight. The pellets were dissolved in 1000µl of Tris-HCl (10mM, pH 8.0). The resulting solution was placed in eppendorf tubes with ammonium sulfate (taking into account that 500µl of Tris correspond 0.165 g of added ammonium sulfate). This mixture was homogenized and left on ice for 15min to allow removal of some impurities (proteins) by precipitation. The samples were centrifuged in an Eppendorf bench-top Centrifuge 5417 R with a rotor F45-30-11 at 18.000g and 4°C for 30min and the supernatant transferred to a new eppendorf tube. Samples were immediately processed in ÄKTA 100 purify system.

4.3 HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC) IN ÄKTA 100 PURIFIER SYSTEM

Previously prepared Bio-Rad Econ-Pac columns, packed with 10cm height hydrophobic interaction resin, Phenyl SepharoseTM 6 Fast Flow was purchased from GE Healthcare (Bio-Rad). The column was connected to an ÄKTA purifier system. Before chromatographic procedures, the columns were always washed with 30ml of Milli-Q water and equilibrated with 30ml of 1.5M ammonium sulfate (in 10mM tris-HCl pH 8.0). The standard purification

protocol used to purify the plasmid DNA was based on [56] and the instrument used was ÄKTA GE Healthcare. A sample of 1ml of pDNA was injected in the column and isocratic elution was performed with 30ml of 1.5M ammonium sulfate (in 10mM Tris-HCl pH 8.0), with recovery of 1.5ml aliquots of flowthrough pDNA fractions. The remaining more hydrophobic molecules (RNA, genomic DNA, denatured pDNA) were eluted by injection of 30ml of 10mM Tris-HCl pH 8.0. The column was washed with 30ml of Milli-Q water and stored with 30ml of PBS and 0.05% sodium azide. Relevant chromatographic fractions, for both flowthrough and eluted peaks were analysed by electrophoresis using 1% of agarose gel. The samples recovered from the procedure, (free RNA) were selected for concentration and diafiltration.

4.4 DIAFILTRATION - AMICON®

pDNA was purified from ammonium sulfate using the Amicon® ultra millipore 30 kDa membranes. The fractions selected were free of RNA and were concentrated. To 2ml of sample volume was put in the Amicon® and centrifuge 15min at 4000g, afterwards and the flow is discharged. The first permeate is again placed for filtration. The process is repeated by placing 2ml of the sample and centrifuged (discharged flow through) until no remaining sample to elute. The diafiltration step was performed by adding 2ml Milli-Q water in the same volume present initially, followed by a centrifugation step using the same settings as before. Recovery of concentrated pDNA was achieved by turning the Amicon® upside down and eluted in 100µl of Milli-Q water, centrifuging it again under the same conditions. The final concentration of pDNA was measured in NanoDrop spectrophotometer at 260_{nm}. A digestion was made as described in section 3.1.5.

5. RESULTS AND DISCUSSION

5.1 OPTIMIZATION OF THE GROWTH OF LAB AT SMALL SCALE AND BENCH SCALE

In order to analyse the growth of LAB cells, the respective cell growth curves at bench scale and small scale with or without pDNA were obtained. It is known that there are some factors which affect the growth of microorganisms, such as, the temperature, pH, agitation, available oxygen concentration, among others. In this case, these strains are anaerobic (although they are aerotolerant), grow with low agitation (100 rpm) and controlled pH of approximately 6.2

It was also verified for these strains that sodium acetate (5g/l) and Tween 80 (1.0g /l) are essential to their growth (depending on trademarks if containing or not). The figure 8 shows the growth curve in falcon tubes and Erlenmeyer flask for these strains. Growth curves were performed with two Erlenmeyer flask and two falcon tubes, such that the last Erlenmeyer flask and falcon was 12 hours delayed in relation to the first. Thus, it was possible to take more points during the growth.

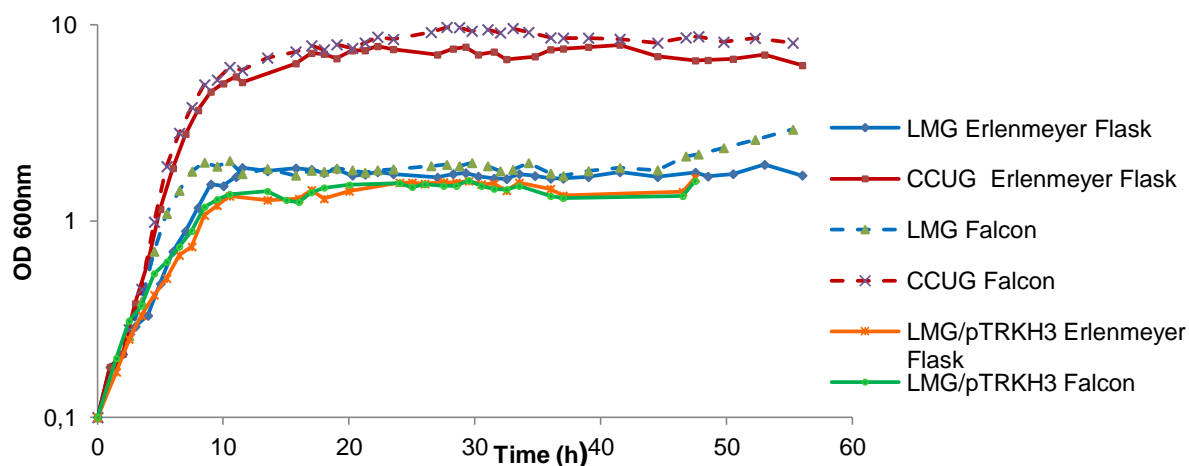


Figure 8 - Growth curve of *L. lactis* LMG 19460 and *L. plantarum* CCUG 61730 harbouring or not pTRKH3

In all strains it was observed that the stationary phase can be maintained for a long time (figure 9), in which biochemical alterations may occur. Although the O.D._{600nm} is the same during this long stationary phase the physiological state is very different and that could affect pDNA production. At both small scale and bench scale, there were no significant differences in maximum O.D._{600nm} they reached. The exponential phase lasts approximately for 4 hours for *L. lactis* LMG 19460 and 6 hours for *L. plantarum* CCUG 61730. For *L. lactis* LMG19460 with pTRKH3 lasts 6 hours, observing slower growth. *L. lactis* LMG 19460 cells without plasmid reach O.D._{600nm} = 3 and O.D._{600nm} = 1.60 with plasmid. The *L. plantarum* CCUG 61730 cells achieve much higher O.D._{600nm} = 10.

The figure 9 shows the specific growth rates for these strains in small scale and bench scale. The maximum specific growth rate (μ_{max}) for both strains was obtained by applying a linear regression to the values in exponential phase. A Napierian logarithm is applied to these values and the maximum specific growth corresponds to the slope of the obtained equation. The *L. lactis* LMG

19460 transformed cells, showed a slight decrease in specific growth rate. It is known, that plasmids may bring a higher metabolic burden to the cell and are likely to reduce the growth rate of the host cell [57].

This happens because cells harboring plasmid

require more energy and nutrient to synthesize more DNA, mRNA and protein than the plasmid free cells [58].

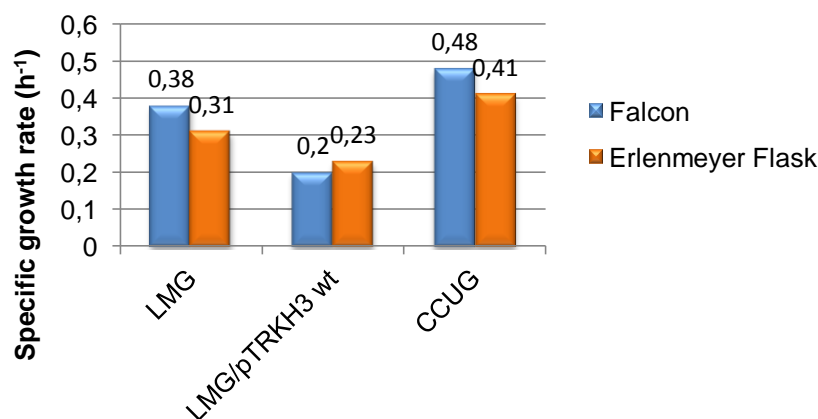


Figure 9 - Specific growth rate of *L. lactis* LMG 19460 and *L. plantarum* CCUG 61730

This can be seen with the slight decrease in the optical density of *L. lactis* LMG 19760 cells, in part due to the sequestration of cellular resources for plasmid replication. The decrease in specific growth rate depends in part on strain, the copy number of pDNA and the nature of the cloning insert. As well as conditions of cell growth and the high levels of oxygen may affect your metabolism [59]. It can be verified at small scale and bench scale, that the growth rate did not affected significantly. The maximum growth rate, for strain *L. plantarum* CCUG 61730 was 0.48 h^{-1} , for *L. lactis* LMG 19460 was 0.38 h^{-1} and *L. lactis* LMG 19460 with pTRKH3, was 0.23 h^{-1} .

5.2 CONSTRUCTIONS OF SEQUENCES OF RBSs

As described above, the choice of RBS was performed using the Anderson library. According to the prediction of this library, both RBS 4 and RBS 5 are described as being stronger for the expression proteins in *E. coli*. However, there may be differences in LAB for expression of REP D and REP E which may or not result in an increased number of copies of pDNA. The figure 10 shows the sequence of the RBS, as well as the codon upstream and downstream and the translation start codon. A few approaches have already been tested at the pAM β 1 origin in order to increase the copy number. It was shown, that inactivation in the copy control region (CopF), led to an increase in the number of copies [37].



Figure 10 - Modification of RBS sequences in pTRKH3

It was also verified that introduction of P_{CT} or T_{DE} mutations in a pDNA, which lacks the CopF repressor, does not cause a further increase in copy number. This may be due to a limiting factor for plasmid replication [31]. In another study was inserted the spac-I promoter upstream of ORF E in the plasmid pAM β 1spac replicated efficiently resulting in about 100 plasmids copies/cell in *Bacillus subtilis* [34].

Thus, in order to test another approach, it was chosen to switch the original RBS sequence of pAM β 1. The modification to RBS 4 was successful however, the complete sequence of the RBS 5 (CAG-GA-CCC), has not been totally achieved. As mentioned before, there are some factors that influence the efficiency of translation. Therefore, the rate at protein synthesis can be different, compared to original sequence (GTG-GA-GTT). It may occur a transient pausing in the translation of the protein sequence that are influenced by the

messenger RNA [42]. Thus, the same protein can be translated at different rates. It was also described that RBS with purine rich sequences A's and G's close to the Shine Dalgarno sequence will lead to high rates of translation initiation [39] as can be observed by the sequence of the switch RBS 4 (GGG-GA-CAA). Then, will be shown the quantification of RBS 4 in some colonies compared to original RBS.

5.3 OPTIMIZATION OF LAB TRANSFORMATION

Plasmid incompatibility may occur if the cells to be transformed already harbour a native plasmid. The native plasmid and the transformed plasmid might compete for components required for their replication. Thus the weaker vector would become unstable or even disappear [19]. Therefore, the strains used in this work were devoid of native plasmids. There are several techniques for cell transformation but among them, electroporation provides the highest efficiency. This is a method that has been successfully employed with a number of bacterial species [50]. The use of a cell wall weakening agent during growth may be advantageous, because stimulate electrocompetence in various bacteria [49]. Incubation with high concentration of sucrose it's known to cause a weakening of the cell wall in several bacteria. Different protocols for electrotransformation, previously described in section 3.2.2 were tested. It was verified that the strain *L. lactis* LMG19460 didn't grow when using the medium of method 1 (G-SGMRS) due to the fact that glycine at this concentration of 2.5% inhibits the formation of cell and affects the growth, in such a way that cells may not complete cellular division. The *L. plantarum* CCUG 61730 strain grew up to $O.D._{600nm} = 1.55$ and $O.D._{600nm} = 0.57$. However, the transformation was not achieved, even varying several parameters, such as the intensity of the shock (either with one pulse or two pulses of 2-500V) and the amount of pDNA (100 ng, 500 ng and 1000 ng). It would be expected that the growth medium could be effective in making cells more competent. However, the 2.5 % of glycine and 0.5M of sucrose (increased osmotic pressure) induced cell wall weakening strategy was shown too much aggressive for *L. lactis* LMG 19460 cells preventing their growth and ineffective for the transformation of *L. plantarum* CCUG 61730 cells.

The second method used was based in a pretreatment of the cells with LiAc and DTT before electroporation. These chemical components (commonly used for transformation of yeast) are reported to enhance the efficiency of transformation [46]. It was demonstrated that the transformation efficiency was higher with these two compounds together, compared omitting one of them [46]. The LAB cells were harvested during the exponential phase and the number of cells (10^9 cells) was controlled as suggested. It was verified that this process did not result for these strains. A test with more cells or an increased electric pulse should be performed.

In Method 3 a simple protocol was tested, involving high concentration of NaCl in the growth medium, acting also as a cell-wall weakening agent. The *L. lactis* LMG 19460 and *L. plantarum* CCUG 61730 cells were grown at a NaCl concentration of 0.7M. The growth in 0.9M NaCl was also tested, but at this concentration the strain *L. lactis* LMG 19460 did not grow, perhaps because of its osmosensitivity. The *L. plantarum* CCUG 61730 cells were grown up to O.D._{600nm} = 0.90, with the same conditions described in table 3 (section 3.2.3). It was verified the failure to obtain transformants. It was also attempted another approach to this protocol, according to the publication [49]. After recovery, the cells were inoculated into a liquid medium (MRS) with sublethal concentration of erythromycin (0.5 µg/ml) overnight. Afterwards, the cellular suspension was plated into MRS agar with 5 µg/ml erythromycin.

Although this approach is described as an alternative for transforming *L. plantarum* and some other species, for our tested strains give no colonies containing pTRKH3.

Table 5 shows the O.D._{600nm} and cell concentrations tested for electroporation of both strains. For the tested conditions, only the *L. lactis* LMG 19460 strain showed transformants.

Table 5: Results of electrotransformation

Strains	O.D. 600nm	Number of cells used in electroporation	Number of Transformants / µg pDNA
<i>L. lactis</i> LMG 19460	0.44	8.5 x10 ⁹ CFU with pTRKH3 wt	3
	0.63	5.89 x10 ⁹ CFU with pTRKH3-RBS4	3
		1.17 x10 ¹⁰ CFU with pTRKH3-RBS 4	16
	0.43	4 x10 ⁹ CFU with pTRKH3-RBS 4	200
		8 x10 ⁹ CFU with pTRKH3-RBS 4	0
<i>L.</i> <i>plantarum</i> CCUG 61730	2.16	9.97 x10 ⁹ CFU with pTRKH3 wt	0
		1.99 x10 ¹⁰ CFU with pTRKH3 wt	0
	2.16	9.97 x10 ⁹ CFU with pTRKH3-RBS 4	0
		1.99 x10 ¹⁰ CFU with pTRKH3-RBS 4	0

In order, to verify the influence of the growth phase in the electrotransformation, *L. lactis* LMG19460 cells were harvested at an O.D._{600nm} = 0.43 (early exponential phase) and O.D._{600nm} = 0.63 (middle exponential phase). In general, many factors can influence the transformation efficiency. Some are linked to the plasmid origin, size, replication mechanisms, whereas others are linked to the characteristics of the strains used like the growth phase of the cells being transformed [48] [50] and effect of agents which weaken the cell wall. It was demonstrated that cells harvested in the middle of exponential growth phase show higher transformation efficiency, when compared to cells recovered in the early exponential phase [50].

By the analyses of the results, it is obtained that there is a variation in the number of transformants that is dependent on the growth phase of the *L. lactis* LMG 19460 cells. In turn, it was reported that too close O.D._{600nm} (0.45-0.50) may lead to differences in the number of transformants [50]. However, the electrotransformation with pTRKH3 carrying the

mutation produced more colonies. It was verified more transformant colonies were achieved at an $O.D._{600nm} = 0.43$. This method proved to be easy, simple and effective for *L. lactis* LMG 19460. It was described as a better method when compared to the other cell wall weakening agents based protocols (lysozyme, glycine or penicillin) [48].

Finally was tested a method 4, where the cells was increased the concentration of glycine up to 8% to grow competent cells. As reported, the number of transformants increases with increasing concentration of glycine [49]. According to the authors, high transformation efficiency was obtained with addition of 8% of glycine to growth medium. In this method, it was decided to not test *L. lactis* LMG 19460 cells, because the glycine (in high concentration) in growth medium inhibited cell growth. The *L. plantarum* CCUG 61730 strain grew up to $O.D._{600nm}=0.38$ and the electroporation was performed as described in attachment 3, table 10, varying cell number and electric pulse. However, this method has proved ineffective to obtain transformant colonies. With this concentration the cell growth seems to have been affected. Thus, the cell growth with a lower glycine concentration of 4% for example, could be tested.

In the tested methods it was also observed that depending on the growth medium of competence cells, growth may be affected, which is reflected in $O.D._{600nm}$ that can reach the cells. It was described that different parameters for the electroporation varies between strains [60]. The number of cells electroporated is important in obtaining the maximum number of transformants [60] and is recommended to use cell concentration in the range of 2×10^8 to 5×10^9 CFU/ml [61]. It was also reported an increase in transformation efficiency with stationary phase cells at high cell concentrations (5×10^{10} CFU/ml) [61]. In another study It was also demonstrated that the number of transformants was proportional to the pDNA concentrations [62] and It was obtained with an expression period of 3 hour best results [60]. The electric pulse also have the impact in the number of transformants obtained. At lower voltage, longer pulses are necessary as well as at the highest voltage, only short pulses should be used [62]. As was demonstrated an inter-relation between all this factors, it can influence transformation efficiency.

5.4 QUANTIFICATION OF RELATIVE PLASMID COPY NUMBER

The relative quantification copy number of plasmid pTRKH3 was performed by using real time PCR (RT-PCR), based on the work of *Carapuça et al.* [55]. Until the date of conclusion of this work, it was not quantified pDNA copy number of the *L. lactis* LMGpTRKH3-RBS 5. It will be done subsequently. Attachment 5 and 6 show all experiments.

The *L. lactis* LMG 19460 cells were harvested at the end of exponential growth phase $O.D._{600nm}=1.01 \pm 0.14$ ($A \pm SD$), where there is no wide variation in the physiological behavior of the cell and consequently in the number of plasmid copies per cell.

To evaluate the relative copy number of pTRKH3, three colonies (3 different transformants) of *L. lactis* LMG19460 with pTRKH3 wt containing the original RBS, (table 6) and 8 different transformants *L. lactis* LMG19460 with pTRKH3-RBS4 (table 6). The figure 11 shows the average of all colonies.

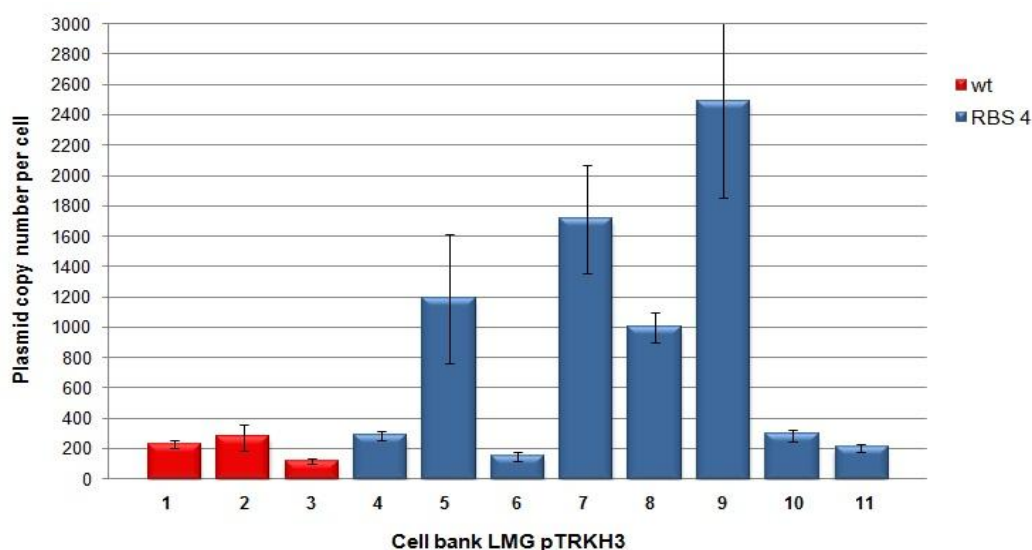


Figure 11 - **Estimated copy number of pTRKH3 for different colonies.** Colonies 1-3: LMG19460 cells electroporated with pTRKH3 wt. Colonies 4-11: LMG19460 cells electroporated with pTRKH3-RBS4. Bar chart shows the averages of each colony of several assays. The error bars represent the standard error (for each cell bank).

The number of copies of the original plasmid pTRKH3 wt (containing the non-modified RBS) varies between (114 and 276 copies per cell), as determined by RT-PCR. Several assays were performed to quantify and also to test the effect of the modifications in the RBS sequence in the plasmid copy number as recommend by [63]. It can thus verify for pTRKH3-RBS4, four colonies which harbor large amounts of pDNA which contain (between 998 and 2490 copies per cell). For these colonies the increase was between 367% and 1065%. Other 4 colonies where there is no wide variation compared to pDNA wt containing (between 149 and 290). These colonies still showed an increased between 34% and 36%. It has been found always a number of copies similar for both experiments with pTRKH3 wt.

Such variation in copy number between colonies (4 to 11), may be due to the ability of some colonies have for supporting a higher number of copies of pDNA. Some colonies may be more metabolically resistant to maintain a high copy number of pDNA. During cell growth it may occurs a significant decrease in plasmid copy number. In order to estimate, if there is a large variation other experiments could be considered. For example, the middle and the end of the exponential phase and early stationary phase could be analyzed. Plasmids with high pDNA copy number, imposes a higher metabolic load to the cell [64]. Thus, there may be some structural change as a result of a physical modification [65]. This can be verified in the colonies 5, 7, 8 and 9 seem to produce large amounts of pDNA (figure 11). The figure 12 shows all growths performed of *L.lactis* LMG 19460 with pTRKH3 wt and pTRKH3-RBS 4.

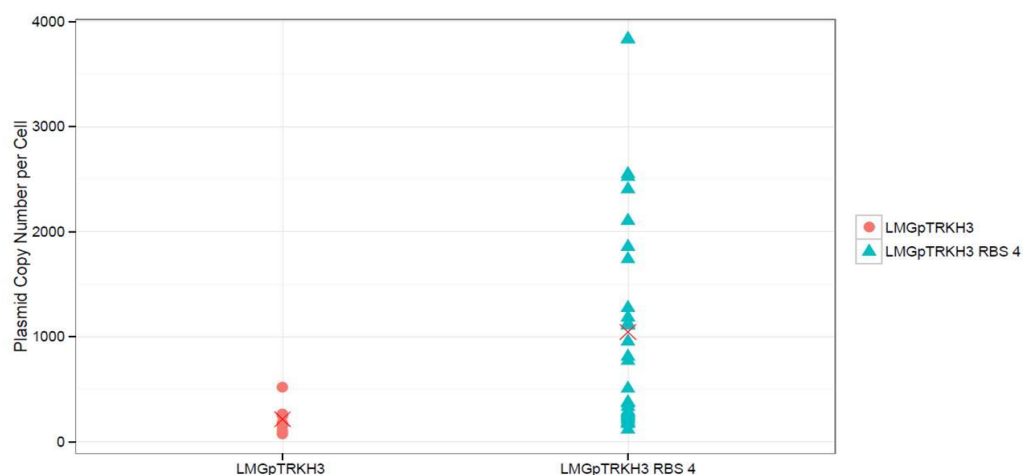


Figure 12 - **Estimated copy number for various assays.** Data displayed represents results from several independent assays with three replicates from each colony. The red balls and blue triangles show the average of all assays. The red X shows the total average. In attachment 6 and 7 can be verified the average $C_{T's}$ value of the total samples analyzed. Scatter plot it was obtained from *R statistical computing software*.

The average of copy number of several growths is high for colonies 5, 7, 8 and 9 compared with the other colonies. It may occur, throughout the generations a gradual loss of the pDNA [57] as a result of high copy number of pDNA. During the exponential phase wherein the metabolic activity of the cell is more intense, pDNA may be a higher burden. Factors such as, composition of medium and rate of cell growth, can also affect the copy number of plasmid [57] [65].

It is described in some cases, that there is some variation in the production of recombinant proteins in *E. coli* clones. Thus, it is possible to exist clones of *L. lactis* LMG 19460 producing more copies of pDNA. The relative value for the number of copies of pTRKH3 calculated may be overestimated. This occurs for both LMG/pTRKH3 wt as for LMG/pTRKH3 – RBS 4. A significant increase in fluorescence (SYBR Green dye) was observed early, as a result of the higher copy number of the pDNA. In this method it was used the SYBR Green dye. This dye binds to any double-stranded DNA and it can also bind to nonspecific double-stranded DNA sequences that could generate false positive signals [66]. Each sample analyzed in the RT-PCR assays, consisting of the same number of cells, resulted in a C_T value (cycle threshold), which corresponds to the number of amplification cycles necessary for the fluorescent signal to surpass the background level (threshold). By analysis of the melting curves, it seems that nonspecific product was not formed. The PCR reactions were analyzed by the melting curves and by gel analysis. Therefore, there may be a nonspecific binding of primers, yielding a PCR product similar to specific fragment of expected size on gel. In order to overcome this, the number of cycles of RT-PCR can be lowered to 25. For an additional analysis it was extracted pDNA by miniprep (results are shown in the next section). Beside the methods used in this study, quantification by HPLC could be performed and for a more precise analysis, a digital PCR may be used.

5.5 COMPLEMENTARY METHOD TO QUANTIFY COPY NUMBER OF pDNA BY MINIPREP

In order to make a complementary analysis to RT-PCR, it were grown colonies 1 and 2 of *L. Lactis* LMG/pTRKH3 wt, colony 5,7,8,9 (higher producers) of LMG / pTRKH3 - RBS 4 and colony 10 of LMG / pTRKH3 - RBS 4. The growths were performed as described in Section 3.2.1 and followed the instructions of the kit protocol (Macherey Nagel Bioanalysis). The final concentration of pDNA was measured in NanoDrop at 260_{nm}. The following table 7 shows the final O.D._{600nm}, the mass of pDNA obtained and corresponding estimated number of copies of pDNA.

Table 6 - Estimated number of copies of pTRKH3 by Miniprep

Cells banks	O.D. _{600nm}	Mass of pDNA after Miniprep	Estimated number of copies of pDNA per cell
Colony 1 LMG/pTRKH3 wt	0.86	13620 ng	177
Colony 2 LMG/pTRKH3 wt	1.14	27180 ng	267
Colony 5 LMG/pTRKH3 – RBS 4	1.20	25200 ng	252
Colony 7 LMG/pTRKH3 – RBS 4	1.39	54960 ng	442
Colony 8 LMG/pTRKH3 – RBS 4	1.10	30120 ng	306
Colony 9 LMG/pTRKH3 – RBS 4	1.17	28800 ng	275
Colony 9 LMG/pTRKH3 – RBS 4	1.03	46800 ng	508
Colony 10 LMG/pTRKH3 – RBS 4	0.78	19800 ng	284
Colony 10 LMG/pTRKH3 – RBS 4	0.99	28800 ng	325

As previously described, it seems to be an increased number of copies of pTRKH3 – RBS 4. It is not certain that the process of lysis by miniprep is completely effective, as this method also presents some limitations. It may happen some saturation of the column during the purification and it may not elute all pDNA. The step of cell lysis may not be completely efficient, requiring for example more than 10mg/ml lysozyme concentrations. It is described that the number of copies of vector pTRKH3 in *streptococcal* and *lactococcal* hosts varies between 45 and 85 copies per cell. A value higher than those was obtained by miniprep (± 222) and by RT-PCR (± 214). Comparing the values of copy number of pTRKH3-RBS4, these results suggest that there is a higher number of copies in *L. lactis* LMG 19460. Moreover, it is known that depending on the strain of *E. coli*, the production of pDNA it can be vary, this may also happen to strains of LAB cells. Therefore, it was also found in a previous study [45], a large variation of the same promoter activity in different strains of LAB.

5.6 CELL Lyses AND CHROMATOGRAPHY

The alkaline lysis method is widely used for pDNA purification from *E. coli*. Therefore, alkaline lysis was the procedure of choice for the first step of pDNA recovery. The purification processes are extremely important, because there are many molecules (genomic DNA, RNA, proteins) that can contaminate the final solution of pDNA [2]. During the alkaline lysis there are some steps which require some care. The mixing is very important, to ensure the lysis of almost all cells in the population, since the surfactant will destabilize the cell wall's integrity.

In addition to this, the mixing should be very gentle to avoid plasmid DNA to be affected by shear and also which can be degraded at high pH values [67]. The next step was the isopropanol precipitation which is used to promote plasmid precipitation. In this work the isopropanol precipitation for 2 h at -20°C yielded larger amounts of pDNA at the end of the purification than precipitation overnight at 4°C. After this step, some contaminants such as RNA and proteins are still remaining. Therefore a precipitation step of these contaminants with 1.5M of ammonium sulfate, was performed. The following step, optimized for plasmid purification from *E. coli*, was hydrophobic interaction chromatography (HIC) using the ÄKTA 100 purifier system.

Before the performance of HIC, it is evident (by 1% agarose gel electrophoresis) the presence of RNA contaminating the pDNA sample (results not shown). HIC separates supercoiled (sc) pDNA from the more hydrophobic single-stranded nucleic acids, RNA, gDNA and endotoxins, [68] (if exploiting the differences in their behavior once exposed to hydrophobic ligands (Phenyl-sepharose resin)) in the chromatographic column. Other plasmid isoforms, such as open circular (oc) pDNA and linear pDNA, which are products of the degradation of sc pDNA, which can occur during even careful pDNA purification processes (due to non-optimal pH, temperature and several centrifugations), but are less efficient in promoting gene expression [68]. These unwanted conformations are caused by single strand nicking, which results in the relaxed form of sc pDNA, the oc pDNA, and double-strand nicking, by restriction endonucleases, which originates linear pDNA. HIC was started by injected the sample after ammonium sulfate clarification into the column connected in ÄKTA 100 purifier. The chromatogram shown in figure 13A indicates a low concentration of pDNA in the eluted fractions. Figure 13B shows pDNA isoforms of the samples collected from HIC from the original purification process.

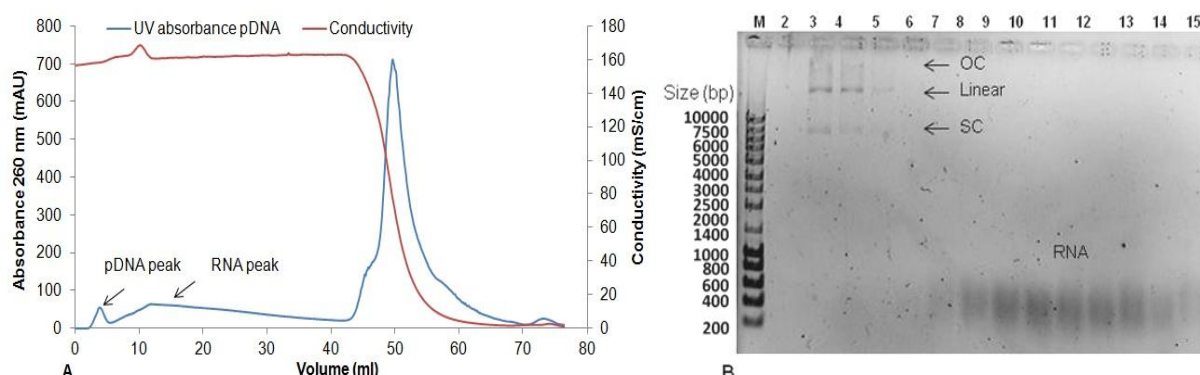


Figure 13 – A: HPLC Chromatogram of pDNA sample from the original purification procedure. B: Agarose gel electrophoresis 1% of the eluted fractions from a purification procedure. It is possible to detect a lower amount of pDNA. Lane M – Molecular-weight marker (NYZ DNA Ladder III, NYZTech). Lanes (2-15) – samples from the first run eluted.

The fractions which contained pDNA were collected and concentrated using the AMICON® 30 kDa membranes. The AMICON® 30kDa membranes are efficiency for desalting and concentrated the sample. Diafiltration, or ultrafiltration, achieves desalting or buffer exchange through the use of centrifugal force to drive small microsolute through a porous membrane. The membrane does not allow macrosolute (larger than the pore size) to pass through. Amicon Ultra devices incorporate a regenerated cellulose membrane to provide maximum sample recovery. The hydrophilic microstructure of membrane ensures the lowest possible adsorption of protein and other macromolecules, and the highest recovery of the sample. Samples of 100µl obtained AMICON were quantified in Nanodrop through spectrophotometry at 260 nm, the final amount eluted contained 11.2 µg.

The quality of the purified pDNA solution and evaluation of the separation of plasmid from impurities was assessed through 1% agarose gel electrophoresis (results nor shown).

In the end the final amount of pDNA was too low. Therefore, a tentative to increase the final pDNA yield was made by adding lysozyme to the lysis step. The rational was the fact that LAB are gram positive bacteria and lysozyme which hydrolyses the peptidoglycan is commonly used in methods for disruption of cell wall, of yeasts and gram positive bacteria.

Comparing the fractions (figure 13A) and the area of peak in the chromatogram of the previous run, the addition of lysozyme, yielded much more plasmid (can be verified by absorbance pDNA pick 600mAU (figure 14A) compared to 50mAU).

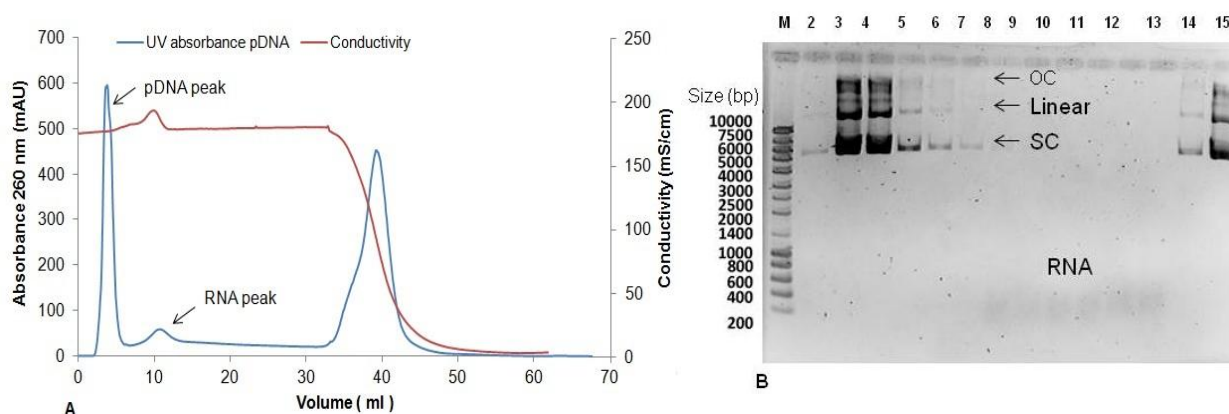


Figure 14 – A: Chromatogram from a purification procedure using lysozyme. B: Agarose gel analysis of the eluted fractions from a purification procedure with lysozyme. M – Ladder III NZYtech; Lines (2-13) – samples from the first run eluted. Lines (14-15) – samples from the second run eluted.

The purification procedure was completed using the AMICON membranes, to remove ammonium sulfate and to concentrate the fractions containing pDNA and no RNA (figure 14). The final amount of pDNA eluted after used AMICON was 368.14 µg. Quality of the purified pDNA was assessed by digestion and agarose gel electrophoresis (as shown in attachment 10). Based on that, it is possible to provide for an increase of 3000 % pDNA with the addition of lysozyme.

6. CONCLUSION

Producing plasmids in LAB cells with a high copy number may not be easy. The LAB cells behave differently from *E. coli* where the production and purification methodologies are well established. In order to adapt and optimize all these techniques, to produce pDNA in LAB, the growth and the purification processes, require several adjustments. From the experiments performed in this work, it can be concluded that, the transformation of the LAB cells, is an important process. Some strains are more easily transformed than others for same the procedures. The method using NaCl was efficient for transformation of *L. lactis* LMG19460. It was also found that the amount of cells required for electrotransformation and the growth phase of cells, strongly influenced the transformation success. The electrotransformation was successful from 4×10^9 to 1.17×10^{10} cells. However, the strain *L. plantarum* CCUG 61730 was unable to give transformants eventually to a cell wall that difficult pDNA uptake even changing some parameters. Additionally, this transformation failure may also be due to the active *endA* gene encoding endonuclease I. It was found that, the type strain *Lactobacillus plantarum* subsp *plantarum* ATCC 14917 has this gene [69]. To confirm that *L. plantarum* CCUG 61730 has this gene, PCR analysis is required.

It was found that lysozyme 10 mg / ml added to the alkaline lysis buffer significantly increased the amount of pDNA (368 µg), comparing to what would happened if we didn't use it (11 µg). However, in order to optimize this process higher concentrations of lysozyme (15 and or 20 mg / ml) or increased incubation periods should be tested. Additionally as plasmid purification was performed with cells from one liter of culture of *L. lactis* LMG/pTRKH3-RBS 4 it would be advantageous to increase the scale to produce more pDNA.

The modified RBS 4 allowed an increase in plasmid copy number up to 332% fold if compared with the non-modified origin of replication present in pTRKH3. To complement this study, other colonies should be analyzed by RT-PCR. These pDNA of the high-producing clones was sequenced and it was confirmed the expected modified sequence. A HPLC analysis of the amount of pDNA produced by these strains is required in order to validate the amounts given by the RT-PCR or for a more precise analysis digital PCR may be used.

This LAB host system may be a safer alternative for the development of DNA vaccines currently produced in *E.coli*. This new application will reinforce other potential applications of LAB for human health, as delivery vehicles and for the production of heterologous proteins.

7. FUTURE PERSPECTIVES

As mentioned before, this thesis was focused on the engineering of pTRKH3 to increase it's copy number in lactic acid bacteria, and thus increment pDNA yield. Additionally, it was necessary to optimize LAB growth and the steps of electrotransformation and pDNA purification. There are thus still many processes that can be optimized in future work. First,

it's needed to confirm that the *endA* gene is active in *L. plantarum* CCUG 61730, and thus would be interesting to knockout *endA* gene. It would be interesting to evaluate the effect of modified origins of replication of pTRKH3 in several LAB strains. Other approaches like promoter P_{DE} replacement may be also tried in order to increase the copy number of pTRKH3.

Optimizing the growth of cells with plasmid in a continuous or fed-batch bioreactor, once there are already some studies for these bacteria applied in the fermentation of food industry, but not in the production of DNA vaccines. On the other hand, several methods of cell disruption could be compared such as, microfluidization, bead milling, sonication and could be good alternatives as they are already being used in biopharmaceuticals and biotechnology processes. It is relevant to optimize the purification process in order to obtain grams of sc pDNA. There are other species and strains of LAB which may contain unknown types of plasmids and it would be interesting to characterize them because they may contain new origins replication.

In the next future, the use of pTRHK3 as a DNA vaccine backbone with therapeutic cloning genes and produced in LAB could be used in animal trials.

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ATTACHMENT

ATTACHMENT 1 – TSS STOCK SOLUTION FOR DH5α COMPETENT CELLS

To prepare TSS stock solution for DH5 α competent cells is necessary adding for 2 ml mixture: 20g/L LB; 5% DMSO; 50mM MgCl₂; 10% PEG 8000 (v/v); pH 6.5.

ATTACHMENT 2 – MRS BROTH PANREAC® CULTIMED

Di-ammonium hydrogen citrate 2.0 g/l, Beef extract 8.0 g/l, Yeast extract 4.0 g/l, D (+) - Glucose 20.0 g/l, Magnesium sulfate 0.2 g/l, Manganese (II) sulfate 0.05 g/l, Bacteriological Peptone 10.0 g/l, Di-Potassium hydrogen phosphate 2.0 g/l, Sodium acetate 5.0 g/l, Tween 80 1.0 g/l, pH6.2.

ATTACHMENT 3 - OTHERS METHODS TESTED FOR ELECTROPORATION

Table 7 - Electroporation conditions of method 1 - G-SGMRS

Method G-SGMRS			
Cells	O.D. _{600nm}	Cell Number and amount of pDNA	Variation in electric Pulse
<i>L. plantarum</i> CCUG 61730	0.57	1.11 x 10 ⁹ CFU in 40 µl and 500 ng pDNA	2-500V and 2x 2-500V
		2.22 x 10 ⁹ CFU in 80 µl and 100 ng pDNA	2-500V
	1.55	10 ⁹ CFU in 100 µl and 1000 ng pDNA	2-000V and 2-500V
		10 ⁹ CFU in 100 µl and 500 ng pDNA	2-500V

Table 8 - Electroporation conditions of method 2 – DTT/LiAc

Method DTT / LiAc			
Cells	O.D. _{600nm}	Cell Number and amount of pDNA	Variation in electric Pulse
<i>L. lactis</i> LMG 19460	0.63	10 ⁹ CFU in 100 µl and 500 ng pDNA	2-500 V
	1.20	10 ⁹ CFU in 100 µl and 1000 ng pDNA	2-500 V
<i>L. Plantarum</i> CCUG 61730	1.27	10 ⁹ CFU in 100 µl and 1000 ng pDNA	2-500 V
	0.51	10 ⁹ CFU in 100 µl and 500 ng pDNA	2-500 V
		1.78 x 10 ⁹ CFU in 100 µl and 1000 ng pDNA	2-500 V

Table 9 - Electroporation conditions of method 4 – 8% Glycine

Method 8% Glycine			
Cells	O.D. _{600nm}	Cell Number and amount of pDNA	Variation in electric Pulse
<i>L. plantarum</i> CCUG 61730	0.35	3.26 x 10 ⁹ CFU in 100 µl and 1000 ng pDNA	2-500V
		4.89 x 10 ⁹ CFU in 100 µl and 1000ng pDNA	2 x 2-500V

ATTACHMENT 4 - PRIMERS FOR SITE DIRECTED MUTAGENESIS

Table 10 - Primers for site directed mutagenesis

Name	Primers	Length pb	Tm
RBS 4	Forward primer 5' – CGTTTGATTGCTTTTTTTGTATTCATTTATA GAAGGGGGACAATGTATGAATCATGATGAATGTAAAA CTTATATAAAA - 3'	79	77
	Reverse primer 3'- TTTTATATAAGTTTTACATTCATCATGATTCATA CATTGTCCCCCTTCTATAAATGAATACAAAAAAA GCAATCAAACG - 5'	79	77
	Forward primer-F3 5' – TTTATATAAGTTTTACATTCATCATGATTCATACA GGCTCCAGCTTCTATAAATGAATACAAAAAAGCAAT CAAACG - 3'	78	78
	Reverse primer-R3 5' – CGTTTGATTGCTTTTTTTGTATTCATTTATAGAAG CTGGAGCCTGTATGAATCATGATGAATGTAAACTTAT ATAAA - 3'	78	78
RBS 5	Forward primer-F2 5' – TTTACATTCATCATGATTCATACAGGGTCCTGCT TCTATAAATGAATACAAAAAAG - 3'	57	73
	Reverse primer-R2 3' – CTTTTTTTGTATTCATTTATAGAAGCAGGACCCT GTATGAATCATGATGAATGTAAA - 5'	57	73

ATTACHMENT 5 - PCR PROGRAME FOR SITE DIRECTED MUTAGENESIS

Reactions were incubated at 95°C for 3 min to activate HotStart DNA polymerase, followed by 30 cycles of 40s at 95°C, 60s at 60°C and 5min at 70°C.

ATTACHMENT 6 – ASSAYS LMG/pTRKH3 (wt)

Table 11 - Assays for RT-PCR - Overview of all cells banks LMG/pTRKH3 wt

	O.D. _{600nm}	Average Ct's	Copy number pDNA	Average	Standard error
Cell bank 1	1.11	15.59	179.8	231.8	26.3
		15.11	250.4		
	0.99	15.80	265.0		
Cell bank 2	1.05	15.72	165.2	276.6	85.6
		14.05	523.0		
		15.05	262.3		
	1.40	15.03	155.8		
Cell bank 3	1.16	16.89	73.4	90.2	16.6
	1	16.35	107.0		

ATTACHMENT 7 - ASSAYS LMG/pTRKH3-RBS 4

Table 12 - Assays for RT-PCR - Overview of all cells banks LMG/pTRKH3 RBS 4

	O.D _{600nm}	Average Ct's	Copy number pDNA	Average	Standard error
Cell bank 4	1.07	15.04	264.1	285.4	29.9
		14.573	364.5		
		14.9	291.0		
	1.02	15.29	222.2		
Cell bank 5	0.63	14.986	274.1	1187.1	423.7
	1	14.967	277.8		
	1.02	12.94	1125.0		
	0.92	12.215	1855.3		
	0.63	11.84	2403.2		
Cell bank 6	1.04	16.23	116.2	149.3	33.1
	1	15.577	182.4		
Cell bank 7	1	12.033	2103.1	1710.2	358.01
		11.77	2522.2		
	0.99	12.965	1105.8		
		12.96	1109.6		
Cell bank 8	0.97	12.866	1183.9	998.2	100.0
		13.18	953.3		
	1.01	13.415	810.6		
		12.76	1273.7		
	1.09	13.29	769.7		
Cell bank 9	0.78	11.163	3834.3	2490.9	636.7
		11.165	3829.0		
	1	11.755	2548.4		
		12.31	1735.6		
	0.99	14.1	505.2		
Cell bank 10	0.97	14.526	376.6	290.0	38.8
		14.705	332.8		
	1.03	15.18	239.8		
	1.05	15.36	211.1		
Cell bank 11	1.03	15.513	190.6	183.4	21.52
		15.12	249.9		
	1.25	15.69	168.7		

ATTACHMENT 8 – CALIBRATION CURVE RT-PCT

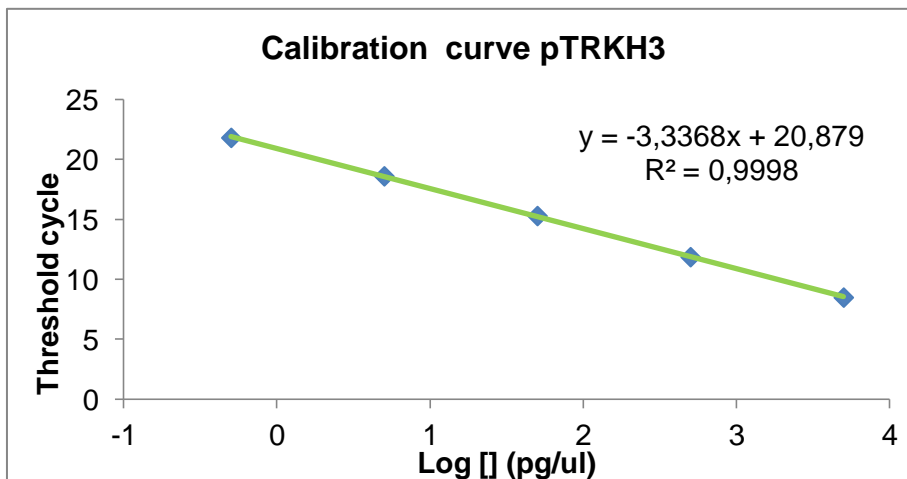


Figure 15 - Standard curve for the relative quantification of plasmid copy number, obtained using pDNA masses ranging from (1pg, 10pg, 100pg, 1000pg, 10000pg). Regression analysis is presented by r^2 values.

ATTACHMENT 9 - AGAROSE GEL 1% - RT-PCR REACTION

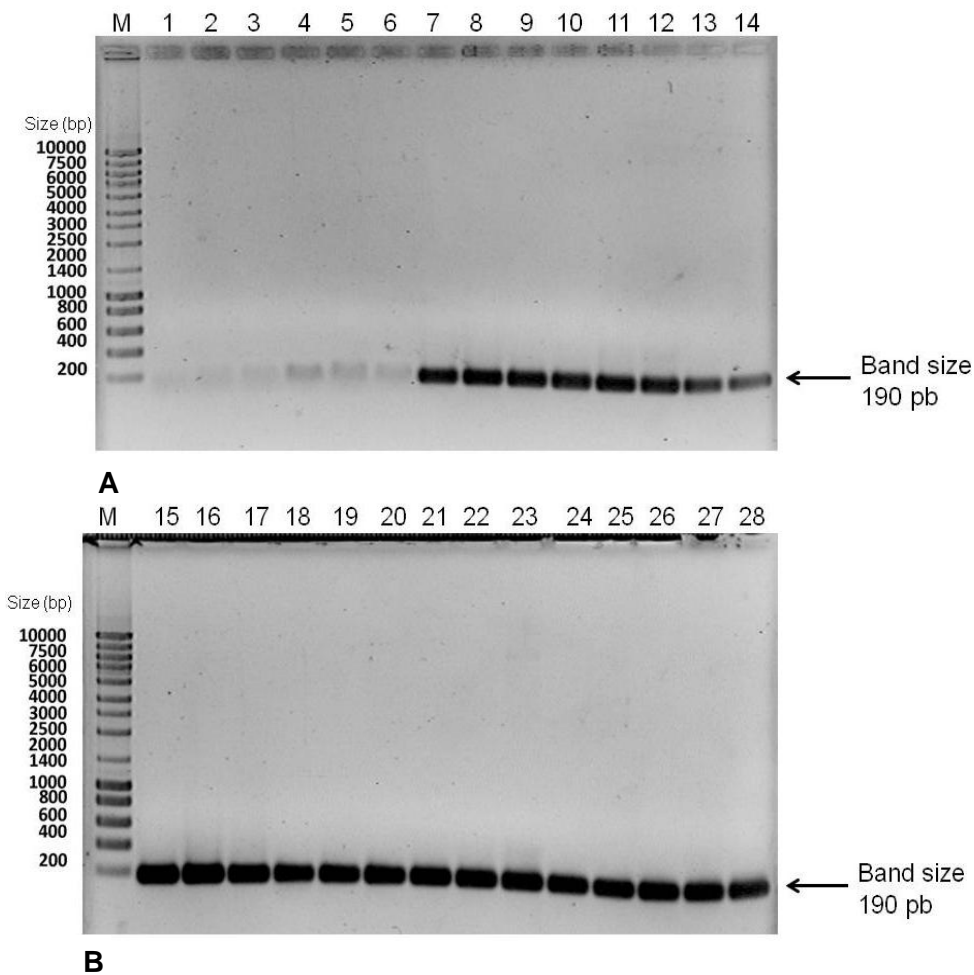


Figure 16 – Confirmation in 1% agarose gel electrophoresis of plasmid pTRKH3 in RT-PCR reaction. A - Lanes (1-6) correspond to controls; Lanes (7-14) correspond to pTRKH3 calibration curve. B - Lanes (15-21) correspond to pTRKH3 calibration curve; Lanes (22-28) correspond to samples; M - Molecular weight marker NYZDNA ladder III (NYZTech).

ATTACHMENT 10 – DIGESTION OF PTRKH3 WITH HIND III

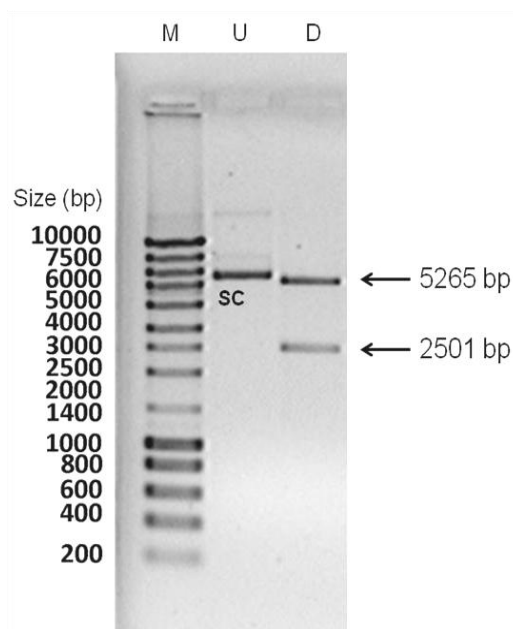


Figure 17 - **Analysis of quality of pTRKH3 by 1% agarose gel electrophoresis.** Lane M: Molecular weight marker (NYZ DNA Ladder III, NYZTech). Lane U: undigested pDNA, it is shown sc pDNA and traces of linear and open circular isoforms; Lane D: Digest pDNA with HIND III.

